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13. ABSTRACT ***(Maximum 200 Words)***

The high incidence of breast cancer and the severity of the disease have created an urgent need for new and innovative forms of treatment. Mucin, the product of the MUC1 gene, has been identified as a breast cancer associated antigen in patients. We use a unique animal model of breast cancer to evaluate immunotherapeutic strategies to treat mice with the disease. Transgenic mice were prepared that express human mucin. The mice are naturally tolerant to human mucin. A malignant mouse breast cancer cell line (410.4) was modified to express human mucin (E3 cells). The cells were then further modified to express B7.1, a co stimulatory molecule required for T cell activation, or to secrete immune-augmenting cytokines. Immunization of MUC1 transgenic mice with E3 cells modified to express B7.1 resulted in the induction of immunity to the breast cancer cells. Also, immunization with E3 cells modified to secrete IL-12 resulted in development of immunity to breast cancer in the MUC1 transgenic mice. Of special significance, MUC1 transgenic mice vaccinated with E3 cells modified to express B7.1 or to secrete IL-12 did not develop autoimmune disease. The immunizations were without harm. In the forthcoming year, we plan to combine these two approaches to define the maximum tumor "load" in MUC1 transgenic mice that can be successfully treated with the modified cells. We also plan to continue our studies determine if the immunizations result in the development of autoimmune disease or are harmful to the MUC1 transgenic mice, and by implication, to breast cancer patients whose tumors express MUC1.

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Edward P. Rolin

Sept 29, 1999

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5. INTRODUCTION

Our investigation was prompted by the finding that polymorphic epithelial mucin (PEM), a product of the human MUC1 gene, is expressed in an altered form by human breast cancer cells (1-3). In its altered form, MUC1 is a tumor associated antigen, uniquely expressed by breast cancer cells, and other types of mucin-producing malignancies. Under appropriate circumstances, MUC1 can be recognized by cytotoxic T lymphocytes. Breast cancer cells that express MUC1 then become the targets of attack by the immune system (4,5). Vaccines that induce immunity to MUC1 may be successful immunotherapeutic agents (6). The long-term objective of our work is to investigate means of increasing the immunogenic properties breast cancer cells that express human mucin. We wish to develop a safe and effective immunotherapeutic approach that can be used in the treatment of breast cancer patients. The development of transgenic mice that express human mucin by one of us (JTP) enables the study to be carried out in experimental animals.

Like breast cancer patients, MUC1 transgenic mice are naturally tolerant to human mucin (7,8). As other cellular constituents, the molecule is viewed as "self" by the animal's immune system. As a model system designed to investigate immunotherapeutic approaches that mimic as closely as possible the treatment of breast cancer in patients, the MUC1 gene was introduced into 410.4 cells, a highly malignant breast cancer cell line of BALB/c origin (H-2^d). After the expression of human MUC1 by the mouse breast cancer cells was confirmed, the cells were then further modified to produce various immune-augmenting cytokines, or to express B7.1, a co stimulatory molecule required for T cell activation. We hypothesized that presentation of MUC1 to the immune system in the microenvironment of immune-augmenting cytokines would stimulate an immune response to breast cancer cells in MUC1 transgenic mice. We also hypothesized that immunization of MUC1 transgenic mice with breast cancer cells modified to express B7.1 would result in the generation of cellular immunity toward the breast cancer cells.

The data described below and presented in greater detail in the accompanying manuscripts indicate the validity of these approaches. In the forthcoming year, we plan to combine these two methods by introducing the gene for B7.1 into breast cancer cells modified for cytokine-secretion that also express human MUC1. The modified cells will be tested for their immunotherapeutic properties in MUC1 transgenic mice with breast cancer.

(6) BODY

Overview of the Progress Report

This investigation was carried out in an animal model of breast cancer. The model mimics as closely as possible the treatment of breast cancer in patients since the mice used in the experiments were modified to express a known human breast cancer antigen (MUC1) and the breast cancer cells used in the experiments were modified to express the same human breast cancer antigen. Thus, both the animals, which are naturally tolerant to human MUC1, and the breast cancer cells express the same human breast cancer antigen.

The objectives of the first and second years of the project have been completed (please see previously submitted progress reports) and significant progress toward the remaining objectives has been achieved. Data presented in three full manuscripts, two of which have been published and one which has been accepted and is "in press," plus one additional full manuscript recently submitted for publication are summarized below. Additional (unpublished) data of work in progress are included in the Appendix. They indicate the following:

1. An immune response to human MUC1 can be induced in MUC1 transgenic mice, naturally tolerant to mucin. The immunogenic properties of human MUC1 expressed by mouse breast cancer cells is markedly enhanced if the cells are modified to express B7.1. The immune response to MUC1 in the immunized mice is mediated by CD8+ T cells

2. An immune response to breast cancer cells that express human MUC1 can be induced in MUC1 transgenic mice if the cells are modified to secrete IL-12. The immunity is systemic, long-term and specific. Like the immunity to breast cancer induced in MUC1 transgenic mice immunized with breast cancer cells modified to express B7.1, the immunity induced by the IL-12-secreting breast cancer cells is mediated by CD8+ T cells.

3. Of special interest, we found no evidence that autoimmunity developed in MUC1 transgenic mice immunized with MUC1-positive breast cancer cells that express B7.1, or secreted IL-12. The animals lived their anticipated life spans. The immunizations appeared to be non toxic and without harm.

4. Immunizations of mice with breast cancer with a vaccine prepared by transfection of DNA from breast cancer cells into a highly immunogenic cell line resulted in immunity to the breast cancer cells and prolongation of survival. This novel method of vaccine preparation has several important advantages that are described, below.

Background

The MUC1 gene codes for a polymorphic membrane associated glycoprotein molecule expressed by epithelial cells that produce mucin. MUC1 is expressed at the apical surfaces of most glandular epithelial cells. It is dramatically up-regulated and overexpressed in breast (and ovarian) carcinoma cells. The glycosylation pattern of MUC1 expressed by mucin-producing carcinoma cells is altered, resulting in the expression of novel T cell epitopes that are potentially immunogenic (9).

The extracellular domain of mucin consists of tandem repeats of twenty amino acids with multiple O-glycans covalently bonded to the amino acid core. In breast and ovarian carcinoma cells, the composition of the carbohydrate side chains is altered, resulting in the exposure of cryptic peptides that are ordinarily hidden in mucin naturally expressed by non

neoplastic cells. Aberrantly expressed breast cancer-associated mucin can become immunogenic and can become the target of cytotoxic T lymphocytes, leading to the rejection of breast cancer cells and the prolongation of survival of tumor-bearing mice. Thus, successful approaches that result in an increase the immunogenic properties of MUC1 could be used to develop a vaccine that might be used in treatment of breast cancer patients.

Increasing the immunogenic properties of breast cancer cells that express human MUC1.
Our initial studies were carried out in transgenic mice injected with a mouse breast cancer cell line (410.4) (10) modified to express human MUC1. (Note-410.4 cells modified to express human MUC1 are designated as E3 cells.) Genes specifying each of several different cytokines known to increase the cells' immunogenic properties were introduced into E3 cells. The immunogenic properties of the cytokine-secreting cells were compared in histocompatible MUC1 transgenic mice.

Progress Report

1. Immunity to breast carcinoma cells modified to express human mucin in transgenic mice that express human mucin.

a. Modification of a mouse breast cancer cell line to express human mucin.
410.4 cells, an adenocarcinoma breast cancer cell line is highly tumorigenic in (non transgenic) syngeneic BALB/c mice. One hundred percent of BALB/c mice injected into the fat pad of the breast with 410.4 cells form progressively growing neoplasms that lead to the animals' death.

A retro viral vector (R1-MUC1-pEMSVscribe) that encoded MUC1, the gene specifying mucin expressed by human breast cancer cells, was used to modify the cells to form human MUC1. The vector also specified an antibiotic resistance gene, used for selection. After

selection, quantitative immunofluorescent staining was used to confirm the expression of MUC1 by the transduced cells. As indicated in Figure 2 of the enclosed manuscript from our laboratory (Development of Immunity to Murine Breast Cancer Cells Modified to Express MUC1, a Human Breast Cancer Antigen, in Transgenic Mice Tolerant to Human MUC1), which has been submitted for publication.

b. Immunohistochemical staining with monoclonal antibodies for MUC1 was used to determine if selected organs and tissues of MUC1 transgenic mice used in the experiments expressed human mucin. A monoclonal antibody (HMFG1) found previously to react with human mucin (11) was used in the study. The results (Fig 1 in the Appendix and in the enclosed manuscript), indicate that MUC1 was expressed on the apical surfaces of cells in the ductal epithelium of distal tubules of the kidney, cells lining bronchioles of the lung and cells in the liver. No effort was made to test all the organs and tissues of the transgenic mice for the expression of MUC1 as these studies were reported previously (12).

c. E3 cells formed progressively growing neoplasms in MUC1 transgenic mice.

MUC1 transgenic mice express human mucin and are naturally tolerant to human mucin. The data presented in the enclosed (unpublished) manuscript (Fig 3), indicate that the time to first appearance of tumor (the latent period) and the rate of tumor growth in MUC1 transgenic mice injected with E3 cells were not significantly different that the latent period and rate of tumor growth in transgenic mice injected with 410.4 cells, a MUC1-negative breast cancer cell line. Thus, mice that were tolerant to human mucin exhibited no resistance to the growth of breast cancer cells modified to express human mucin. In contrast, the latent period and rate of growth of E3 cells in (non transgenic) BALB/c mice was significantly less than that of 410.4 cells, as presented in Figure 3. Human MUC1 is weakly antigenic in BALB/c mice.

6. Cytokine secretion by E3 cells transduced with retroviral vectors encoding cytokine genes.

Cytokine-secretion by cancer cells is known to augment the cells' immunogenic properties (13-15). Several immune augmenting cytokines were evaluated to determine if cytokine-secretion by E3 cells increased the cells' immunogenic properties in MUC1 transgenic mice. As a first step, retro viral vectors were used to modify the cells to secrete IL-2, IL-4, IFN-g or IL-12. The results presented in the enclosed manuscript confirmed the presence of the relevant cytokine in the culture supernatants of the transduced cells. Passage of the cells in selection medium maintained cytokine-secretion by the transduced cells for more than three months of continuous culture.

7. Secretion of IL-12 by E3 inhibited the cells' tumorigenic properties in MUC1 transgenic mice.

To determine if cytokine-secretion by the cells affected their tumorigenic properties, E3 cells modified to secrete IL-2, IL-4, IFN-g or IL-12 were injected into the fat pad of the breast of MUC1 transgenic mice. As a control, the mice were injected into the fat pad of the breast with an equivalent number of non cytokine-secreting E3 cells transduced with a vector (pZipNeoSV(X) that conferred G418 resistance but did not specify a cytokine gene. The results presented in detail in Figure 5 of the enclosed (unpublished) manuscript indicated that the latent period in the group of MUC1 transgenic mice injected with E3 cells modified to secrete IL-12 (E3-IL-12 cells) was significantly prolonged, relative to that of mice in any of the other groups ($P < .01$). Six of the 8 MUC1 transgenic mice injected with E3-IL-12 cells failed to form tumors and appeared to have rejected the highly malignant breast cancer cells. Two of the mice injected with E3-IL-12 cells formed slowly growing tumors at the injection site. The median survival time (MST) of these mice, approximately 100 days, was significantly ($p < .001$) longer than the MST of mice in any of the other groups including mice injected with E3 cells modified to secrete any of the other cytokines tested. H and E

staining of tissue sections taken from the site of injection of mice injected with E3-IL-12 cells revealed an intense inflammatory infiltrate consistent with the rejection of the cytokine-secreting cells (Figure 1, panels a, b and c, included in the Appendix.).

8. MUC1 transgenic mice that rejected the IL-12-secreting E3 cells developed immunity to breast cancer cells modified to express human mucin.

Tumors failed to form in six of the eight MUC1 transgenic mice injected with IL-12-secreting E3 cells. To determine if the mice that rejected the IL-12-secreting cells developed immunity toward E3 cells, the surviving mice were injected with E3 cells 71 days after the injection of E3-IL-12 cells. As indicated (Fig 6 in the enclosed (unpublished) manuscript), none of these animals formed tumors. Under similar conditions, all of the naïve MUC1 transgenic mice injected with (non cytokine-secreting) E3 cells developed progressively growing neoplasms at the injection site and died from breast cancer.

Thus, the immunogenic properties of E3 cells modified to secrete IL-12 clearly exceeded those of E3 cells modified to secrete any of the other cytokines.

9. Resistance to breast cancer in MUC1 transgenic mice immunized with E3-IL-12 cells was mediated by cellular immune mechanisms.

The failure of E3 cells to grow in MUC1 transgenic mice that rejected E3-IL-12 cells suggested that the resistance was mediated by immune mechanisms. An immunoassay based on the release of cytokine (interferon-g (IFN-g) by antigen-stimulated spleen and lymph node cells was used to investigate this question (16). The results (Table 1 in the enclosed (unpublished) manuscript) indicated that the titers of IFN-g in the culture supernatants of spleen/lymph node cells from MUC1 transgenic mice immunized with E3-IL-12 cells that were co-incubated with E3 cells, or 410.4 cells, were significantly higher than the titers of

IFN-g in the culture supernatants of cells co-incubated with B16 cells, an immunologically unrelated mouse neoplasm. IFN-g was undetectable in the supernatants of spleen/lymph node cell cultures that were incubated without the addition of the tumor cells. Analogous findings were observed if the culture supernatants were analyzed for the presence of MIP1a, a chemokine. The titers of MIP1a in culture supernatants of spleen/lymph node cell suspensions from mice immunized with E3-IL-12 cells co-incubated with E3 cells or 410.4 cells were significantly higher than found in culture supernatants from cell suspensions co-incubated with B16 cells, or from cell suspensions incubated alone. Thus, cellular anti breast cancer immune mechanisms were activated in MUC1 mice immunized with MUC1-positive mouse breast cancer cells modified to secrete IL-12.

An immunofluoresence assay was used to determine if antibodies reactive with E3 cells were present in the sera of MUC1 transgenic mice immunized with E3-IL-12 cells. The results (Table 2 in the enclosed (unpublished) manuscript) indicated that if antibodies reactive with E3 cells were present and that IgG was the major immunoglobulin class reactive with E3 cells in mice immunized with E3-IL-12 cells.

10. Expression of B7.1 by E3 cells augmented the cells' immunogenic properties in MUC1 transgenic mice.

As noted previously, the MUC1 gene is expressed on the apical surfaces of normal glandular epithelial cells of the breast that produce mucin. Mucin is overexpressed and aberrantly glycosylated in breast cancer cells.

The development of a human MUC1 transgenic mouse that expresses MUC1 along with the genetic modification of 410.4 breast cancer cells to express human MUC1 (E3 cells) provides a unique opportunity to investigate the effect of expression of B7.1 by E3 cells on the cells'

immunogenic properties. B7.1 is a co stimulatory molecule required for T cell activation (17). It is constitutively expressed by dendritic cells and other types of antigen presenting cells.

Prior reports indicate that the introduction of a gene for co stimulatory molecules such as B7.1 into tumor cells can enhance the cells' immunogenic properties and thus reduce their tumorigenicity. To determine if the expression of B7.1 by E3 cells affected the cells' immunogenic properties in MUC1 transgenic mice, E3 cells were co transfected a cDNA encoding murine B7.1 contained in the plasmid πLN along with a vector that conferred resistance to G418, used for selection. After confirmation of the expression of B7.1 by the breast cancer cells, the cells were tested for their immunogenic and therapeutic properties in MUC1 transgenic mice. The results are summarized in the enclosed (published) manuscript from our laboratory entitled, "Expression of B7.1 in a MUC1-expressing mouse mammary epithelial tumor cell line inhibits tumorigenicity but does not induce autoimmunity in MUC1 transgenic mice," by M. Smith, JM, Burchell, R Graham, EP Cohen and J Taylor-Papadimitriou. Immunology 97: 648-655, 1999. A copy is included in the Appendix.

The results may be summarized as follows:

The expression of B7.1 by E3 cells augmented the cells' immunogenic properties as indicated by a dramatic inhibition of tumor growth in MUC1 transgenic mice injected with the modified cells. The anti tumor activity was mediated by CD+ and CD4+ T cells.
Of special interest, MUC1 transgenic mice immunized with MUC1-expressing breast cancer cells modified to express B7.1 (or to secrete IL-12), did not exhibit signs of autoimmunity. An examination of various organs and tissues from the mice failed to reveal signs of disease. The mice lived their normal life spans without apparent toxic effects.

We believe that the lack of an autoimmune response in MUC1 transgenic mice immunized with MUC1-positive breast cancer cells modified to secrete IL-12, or to express B7.1, is a

most important finding. The data indicate that immunotherapy of breast cancer patients who neoplasms express MUC1 with a vaccine prepared by modification of breast cancer cells to secrete IL-12, or to express B7.1 is not likely to induce an autoimmune disease. Tumor regression in the immunized mice suggests that an analogous form of treatment breast cancer patients might offer significant benefit to breast cancer patients.

Treatment of breast cancer with DNA-based vaccines.

In addition to our studies of the immunotherapeutic properties of breast cancer cells modified to express B7.1, or to secrete IL-12, as described above, we are also engaged in complementary studies designed to investigate the immunotherapeutic properties of a DNA-based vaccine in the treatment of mice with breast cancer. The underlying rationale for this approach is that like other types of malignant cells, breast cancer cells are genetically unstable. As a consequence, the cells may specify more than one breast cancer antigen. To "capture" these additional, undefined antigens, we transfer DNA from mouse breast cancer cells into a highly immunogenic cell line. Genes specifying breast cancer antigens are expressed in an immunogenic form by the transfected cells, as indicated by the finding that mice immunized with cells transfected with breast cancer DNA are resistant to challenge with breast cancer cells (please see enclosed (published) manuscript from our laboratory entitled, "Immunity to Breast Cancer in Mice Immunized with Semi-Allogeneic Fibroblasts Transfected with DNA from Breast Cancer Cells." Journal of Immunology, 162: 6934-6941, 1999. A copy is included in the Appendix).

The DNA-based vaccine was prepared by transfection of a mouse fibroblast cell line (LM) with DNA from an adenocarcinoma of the breast that formed spontaneously in a C3H/He mouse. The fibroblasts were first modified to secrete interleukin-2. A retrovirus (pZipNeoSV-IL-2) was used for this purpose. After confirmation that the cells were secreting IL-2, the fibroblasts were further modified to express H-2K^b-determinants. This

step was taken because H-2K^b-determinants are allogeneic in C3H/He mice, the original source of the breast cancer cells. Allogeneic determinants are known to act as immune-adjuvants. The modified fibroblasts were then co-transfected with DNA from a spontaneous breast neoplasm that arose in a C3H/He mouse and tested for their immunotherapeutic properties against the growth of the breast cancer cells in the same strain of mice. The results indicated that mice with breast cancer treated solely by immunization with LM fibroblasts transfected with DNA from the breast neoplasm survived significantly ($P < .01$) longer than mice in various control groups including mice with breast cancer treated by injection with non DNA transfected fibroblasts or fibroblasts transfected with DNA from a heterologous tumor (B16 melanoma). Similar beneficial effects were observed in C57BL/6 mice injected with a syngeneic breast cancer cell line (EO771) and the modified fibroblasts transfected with DNA from E0771 cells. The immunity was mediated by CD8+ T cells since immunized mice depleted of CD8+ cells failed to resist tumor growth.

Figure 2 included in the Appendix illustrates the promise of this approach.

Autoimmunity fails to develop in mice treated with DNA-based vaccines.

Like the treatment of MUC1 transgenic mice with breast cancer cells modified to express B7.1, or to secrete IL-12, the development of an autoimmune disease in mice with breast cancer treated by immunization with a DNA-based vaccine is of concern. Both tumor associated as well as "normal" antigens will be expressed by the DNA-transfected cells. (Other approaches that reply on the transfer of RNA or cDNA from tumor cells into antigen presenting cells, or the "feeding" of peptides eluted from tumor cells to dendritic cells are subject to similar concerns. "Normal" antigens are expressed in an "immunogenic" form by the antigen presenting cells.)

To determine if mice immunized with the DNA-based vaccine exhibited evidence of autoimmunity, various organs and tissues of the immunized mice were analyzed for evidence

of lymphoid cell infiltrates. The results are presented in the accompanying Fig 3, included in the Appendix. They indicate that there were no histologic changes in the immunized mice suggestive of autoimmunity. Tumor-free mice injected with the DNA-transfected cells exhibited no ill effects from the vaccine and survived indefinitely.

(7) KEY RESEARCH ACCOMPLISHMENTS

1. A mouse breast cancer cell line (410.4) was successfully modified to express MUC1, a previously described human breast cancer antigen and to secrete IL-2, IL-4, IFN-g or IL-12, immune augmenting cytokines. The cytokine-secreting cells were tested for their immunotherapeutic properties in histocompatible transgenic mice that expressed MUC1 as "self" and are naturally tolerant to the molecule.

2. The immunogenic properties of the highly malignant breast cancer cells modified to express MUC1 and to secrete IL-12 exceeded those of MUC1-positive breast cancer cells modified to secrete IL-2, IL-4 or IFN-g in MUC1 transgenic mice. Of significance, there was no evidence that autoimmunity developed in MUC1 transgenic mice immunized with the IL-12 secreting cells.

3. Modification of breast cancer cells to express MUC1 and to express B7.1, a co stimulatory molecule, dramatically reduced the cells' tumorigenic properties in MUC1 transgenic mice. The latent period following tumor injection was prolonged and the mice survived significantly longer than mice in various control groups.

4. The growth of tumor in immune-deficient nu/nu (nude) mice injected with E3 cells modified to express B7.1 was the same as the growth of tumor in nude mice injected with E3 cells that did not express B7.1, suggesting that cytotoxic T lymphocytes were involved in mediating tumor rejection in MUC1 transgenic mice.

5. Both CD4+ and CD8+ cells were required to mediate resistance to the modified breast cancer cells in the MUC1 transgenic mice.

6. Autoimmune disease was not detected in transgenic mice injected with E3 cells modified to express B7.1. There was no evidence of lymphocytic infiltration or organ degeneration in MUC1 transgenic mice that were injected with E3 cells modified to express B7.1.

7. A vaccine prepared by transfer of DNA from adenocarcinoma of the breast that formed spontaneously in a C3H/He mouse was effective in the treatment of mice with breast cancer.

(8) Reportable outcomes

A list of published manuscripts, manuscripts submitted for publication and manuscripts that have been accepted for publication and are "in press" during the 1998-99 period of grant support is as follows:

1. de Zoeten, E., Carr-Brendel, V., Markovic, D., Taylor-Papadimitriou and Cohen. E.P. Immunity to Breast Cancer in Mice Immunized with Semi-Allogeneic Fibroblasts Transfected with DNA from Breast Cancer Cells. Journal of Immunology, 162: 6934-6941, 1999

2. Carr-Brendel, V, Markovic, D., Smith, M., Taylor-Papadimitriou, J and Cohen, EP. Immunity to Breast cancer in Mice Immunized with X-Irradiated Breast Cancer Cells Modified to Secrete IL-12. J Immunotherapy, "in press"

3. Smith, M., Burchell J.M., Graham, R., Cohen, E.P. and Taylor-Papadimitriou, J. Expression of B7.1 in a MUC1 expressing mouse mammary epithelial tumor cell line overcomes tolerance but does not induce autoimmunity in MUC1 transgenic mice. Immunology 97: 648-655, 1999.

4. Development of Immunity to Murine Breast Cancer Cells Modified to Express MUC1, a Human Breast Cancer Antigen, in Transgenic Mice Tolerant to Human MUC1, Carr-Brendel, V., Markovic, D., Ferrer, K., Smith, M., Taylor-Papadimitriou, J., Cohen, E.P. submitted

(9) Conclusions

We conclude the following:

1. The immunotherapeutic properties of breast cancer cells that express MUC1, a human breast cancer antigen, are enhanced in MUC1 transgenic mice if the cells are modified to secrete IL-12.

2. A vaccine prepared by modifying breast cancer cells that express MUC1 are also enhanced if the cells are modified to express B7.1, a co stimulatory molecule.

3. Immunizations of mice with the breast cancer cells that express B7.1 or secrete IL-12 does not induce autoimmunity in MUC1 transgenic mice.

4. A breast cancer vaccine that prolongs the survival of mice with breast cancer can be prepared by transfer of DNA from breast neoplasms that arise spontaneously in C3H/He mice. The vaccine is non toxic.

"So what"

The treatment of breast cancer patients has not progressed significantly in many years. Most patients whose tumors have spread beyond the primary site eventually die of their disease. New and innovative types of treatment that differ from prior forms of therapy need to be investigated and eventually brought to clinical trial.

Immune therapy based on tumor cell vaccines is of promise. The treatment is selective in the sense that only the patient's malignant cells are killed. It is non toxic and appears to be without harm.

Two approaches toward vaccine development were evaluated using funds provided by the Department of Defense. Using a unique mouse model of human breast cancer, breast cancer cells that expressed MUC1, modified to secrete IL-12, were found to be highly immunogenic and non toxic to MUC1 transgenic mice with breast cancer. An analogous form of vaccine may be evaluated in breast cancer patients, using breast cancer cells, modified for IL-12-secretion, as a vaccine. Analogous results were obtained if the cells were modified to express B7.1, a co stimulatory molecule. Again, the immunizations were found to be non toxic. An autoimmune disease did not develop in spite of the fact that the vaccine included many "normal" antigens..

The second approach involved the transfer of DNA from either of two adenocarcinomas that arose spontaneously in C3H/He mice into a highly immunogenic cell line. The use of cells from a primary breast carcinoma (rather than a tumor cell line) closely resembled the disease in patients. Immunizations of mice with breast cancer with the DNA-based vaccine resulted in generalized, long-term immunity to the tumor, and prolongation of survival. This approach has an important practical advantage. Since the transferred DNA is integrated into the genome of the recipient cells, and is replicated as the cells divide, a sufficient quantity of vaccine can be prepared from small surgical specimens. A needle biopsy of tumor could provide enough DNA for this purpose.

Taken together, these findings are the first demonstrations, in a system that closely mimics breast cancer in patients, that a non toxic tumor vaccine can be conveniently prepared that is of use in the treatment of the disease. Immunizations of mice highly susceptible to breast

cancer resulted in the induction of immunity to the breast cancer cells and prolongation of survival.

Plans for future work

We plan two main initiatives in the forthcoming year to further define and expand our previous findings.

For the first, we will modify E3 cells to secrete IL-12 and to express B7.1 The modified cells will be tested in MUC1 transgenic with established breast neoplasms for their immunotherapeutic properties, and compared with the immunotherapeutic properties of E3 modified to secrete IL-12 or to express B7.1 alone. Mice bearing various numbers of tumor cells will be tested so as to define as closely as possible the maximum immunotherapeutic properties of the modified cells. As previously, we will monitor the mice carefully for evidence of autoimmune disease or other types of toxicity to the breast cancer vaccine.

We are also continuing our development of a construct that specifically restricts expression of B7.1 to breast cancer cells. We have defined the region of the MUC1 promoter that is responsible for tissue and tumor specific expression in vivo and have developed a construct in which the B7.1 molecule is driven by the MUC1 promoter.

We are continuing our work on the development of transgenic mice that are homozygously transgenic for MUC1 and are crossing these mice with a mouse that is transgenic for the polyoma T gene. Our preliminary data thus far indicate that these mice develop breast neoplasms within weeks of birth.

(10) References

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Legends

Figure 1.

Immunohistochemical and H and E staining of various tissues of MUC1 transgenic mice stained with an antibody (HFMG-1) reactive with human MUC1.

Legend:

(a)= Inflammatory infiltrate at the site of injection of E3-IL-12 cells into breast tissue of MUC1 transgenic mice. H and E section (250X)

(b) = Inflammatory infiltrate at the site of injection of E3-IL-12 cells into breast tissue of MUC1 transgenic mice. H and E section (400X)

(c)= Inflammatory infiltrate and a mucin positive breast cancer cell (arrow) in breast tissue from MUC1 transgenic mice injected with E3-IL-12 cells. Immunohistochemical staining (400X)

(d)= Immunohistochemical staining of portal tracts in the liver of MUC1 transgenic mice with immunostaining localized to bile duct epithelium (400X)

(e)= Immunohistochemical staining of portal tracts in the liver of MUC1 transgenic mice, showing strong luminal surface staining (1000X)

(f)= Immunohistochemical staining of bronchial epithelium of MUC1 transgenic mice (1000X)

(g) = Immunohistochemical staining of bronchial epithelium of MUC1 transgenic mice (400X)

(h)= Immunohistochemical staining of renal cortex of MUC1 transgenic showing reaction with cells in the distal convoluted tubules (400X)

(i) = Immunohistochemical staining of renal cortex of MUC1 transgenic mice showing reaction with cells in the distal convoluted tubules (1000X)

For the immunohistochemical staining, the tissues were fixed in methanol:chloroform:acetic acid, (60:30:10) and paraffin embedded for sectioning. Dewaxed paraffin sections were incubated with 50% fetal calf serum in PBS for 30 min to prevent non specific binding of antibodies. The blocking solution was then removed and replaced by neat hybridoma culture supernatant, followed by incubation at room temperature for 60 min. After three 5 min washes with PBS, the sections were incubated for 1 hr. with peroxidase-conjugated rabbit anti mouse immunoglobulin antiserum (Dako; diluted 1:50 in PBS with 15% fetal calf serum). The slides were then washed three times with PBS. The substrate solution consisting of .03 percent hydrogen peroxide in PBS and 1 mg/ml diaminobenzidine (Sigma) was added and the reaction was allowed to continue for 5 to 8 min. After final washing with PBS, the slide were counterstained with H and E and mounted.

Figure 2.

Expression of MUC1 by mouse breast cancer cells (410.4) transfected with a plasmid encoding the MUC-1 gene (E3 cells).

Legend: 1 x 10^6 E3 cells or 410.4 cells were incubated for 1 hr. at 4°C with SM-3 (A) or HFMG-1(B) antibodies or isotype control antibodies. After incubation with anti-mouse-FITC antibodies and washing as described in Materials and Methods section, the cells were analyzed for fluorescent staining by flow cytofluorography. {A} Light line, cells incubated without the primary antibody, dotted line, 410.4 cells incubated with SM-3 antibodies, bold line, E3 cells incubated with SM-3 antibodies. {B} Light line, cells incubated without the primary antibody, dotted line, 410.4 cells incubated with HFMG-1 antibodies, bold line, E3 cells incubated with HFMG-1 antibodies.

Figure 3.

Tumor growth in BALB/c mice injected into the fat pad of the breast with 410.4 cells or E3 cells.

Legend: Naive BALB/c female mice between 12 to 16 weeks were injected into the fat pad of the breast with 1 x 10^6 E3 cells (B) in a total volume of 200 ul growth medium or, for comparison, with 1 x 10^6 410.4 cells (A) in 200 ul growth medium. Two dimensional tumor measurements were performed and the volume of the tumor was calculated as described in the Materials and Methods section. Each line represents tumor growth in an individual mouse. The endpoint of the line represents the death of the mouse.

Figure 4.

Tumor growth in MUC-1 transgenic mice injected into the fat pad of the breast with 410.4 or E3 cells.

Legend: Tumor growth was measured in naive MUC-1 transgenic mice injected into the fat pad of the breast with 1 x 10^6 E3 cells (B) in a total volume of 200 ul growth medium. For comparison, naive MUC-1 transgenic mice were injected with 1 x 10^6 410.4 cells (A) in 200 ul growth medium. Two dimensional tumor measurements were performed and the volume of the tumor was calculated as described in the Materials and Methods section. Each line represents tumor growth in an individual mouse. The endpoint of the line represents the death of the mouse.

Figure 5.

Tumor growth in MUC1 transgenic mice injected into the fat pad of the breast with E3 cells modified for cytokine-secretion.

Legend: Naive MUC1 transgenic female mice between 12 and 16 weeks of age were injected into the fat pad of the breast with 1 x 10^6 E3 cells modified for cytokine secretion. For comparison, the mice were injected with an equivalent number of unmodified E3 cells or E3 cells transfected with a vector (pZiPNeoSV-X) that specified a neomycin-resistance gene but did not encode a cytokine gene. Each cell-type was suspended in 200 ul of growth medium for the injection. Two dimensional tumor measurements were performed and the volume of the tumor was calculated as described in the Materials and Methods section. Each line represents tumor growth in an individual mouse. The endpoint of the line represents the death of the mouse. There were eight mice in each group.

Figure 6.

Tumor growth in MUC-1 transgenic mice surviving a prior injection of E3-IL-12 cells injected subsequently with E3 cells.

Legend: MUC1 transgenic female mice between 12 and 16 weeks of age were injected into the fat pad of the breast with 1 x 10^6 E3-IL-12 cells suspended in 200 ul of growth medium. Seventy one days later, the mice were injected a second time into the fat pad of the contralateral breast with 1 x 10^6 viable E3 cells suspended in 200 ul of growth medium (■). As a control, naive MUC1 transgenic female mice of the same age were injected into the fat pad of the

breast with an equivalent number of E3 cells suspended in 200 ul of growth medium (□).

Table 1.

Cytokine-release from a suspension of spleen and lymph node cells from MUC1 transgenic mice injected with E3-IL-12 cells co incubated with X-irradiated E3 cells.

Legend: MUC1 transgenic female mice between 12 to 16 weeks of age were injected into the fat pad of the breast with 1 X 10^6 E3-IL-12 cells suspended in 200 ul of growth medium. Fourteen days later, the animals were sacrificed and a pooled cell suspension was prepared from the spleen and lymph nodes of individual mice. The nucleated cells (Histopaque) were co incubated for 24 hrs. with X-irradiated (5000 rads from a ^{60}Co source) E3 cells. The ratio of spleen/lymph node cells to E3 cells was 3:1. At the end of the incubation, the culture supernatants were analyzed by ELISA for the presence of IFN-g or MIP1-α.

As controls, X-irradiated 410.4 cells or X-irradiated B16 cells were substituted for the E3 cells, or the spleen/lymph node cell suspensions were incubated alone. The results represent the mean ± SD cytokine release from each of three individual mice.

Table 2.

Isotype of antibodies to E3 cells in MUC1 transgenic mice immunized with E3-IL-12 cells.

Legend: Naive MUC1 transgenic female mice between 12 and 16 weeks of age were injected into the fat pad of the breast with $1x10^6$ E3-IL-12 cells suspended in 200 ul of growth medium. As controls, naïve MUC1 transgenic mice were injected with an equivalent number of E3 cells, or 410.4 cells suspended in 200 ul of growth medium. The mice in each group were bled thirty days later. A 1:100 dilution of the pooled sera was incubated with 5 x 10^5 E3 cells for 45 min. at 4^0. At the end of the incubation, the cells were washed and then incubated for 45 min. at 4^0 with FITC-conjugated goat anti mouse IgG, anti mouse IgM or anti mouse IgA serum. The intensity of immunofluorescent staining was measured by flow cytofluorography using a FACSCalibur cytofluorograph (Becton-Dickinson). There were three mice in each group.

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Development of Immunity to Murine Breast Cancer Cells Modified to Express MUC1, a Human Breast Cancer Antigen, in Transgenic Mice Tolerant to Human MUC1.

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Running title: Immunity to breast cancer in transgenic mice.

ABSTRACT

The high incidence of breast cancer in woman and the severity of the disease have stimulated a need for improved and novel forms of therapy. Polymorphic epithelial mucin (PEM), the product of the MUC1 gene, has been identified as a breast cancer-associated antigen in breast cancer patients. The gene has been cloned and sequenced. Transgenic mice have been prepared that express human mucin and are naturally tolerant to the molecule, providing a unique opportunity to investigate immunotherapeutic strategies in experimental animals that might eventually be applied to breast cancer patients. A cell line (410.4) derived from a mouse mammary adenocarcinoma that arose in a BALB/c mouse was transduced with a retroviral vector (R1-MUC1-pEMSVscribe) that encoded MUC1. After confirmation of the expression of MUC1, the cells (E3) were further modified by transduction with retroviral vectors encoding IL-2, IL-4, interferon-g or IL-12 to evaluate the effect of cytokine-secretion on the cells' immunogenic properties in the MUC1 transgenic mice. The results indicated that modification of the breast cancer cells to secrete IL-12 reduced and at times eliminated the cells' tumorigenic growth properties. Under similar circumstances, progressively growing tumors formed in MUC1 transgenic mice injected with unmodified E3 cells or with E3 cells modified to secrete IL-2, IL-4 or IFN-g. Immunity to breast cancer developed in MUC1 transgenic mice that rejected the IL-12-secreting E3 cells since the animals were resistant to challenge with (non cytokine-secreting) E3 cells. In vitro analyses confirmed the presence of T cell-mediated cytotoxicity toward the breast cancer cells in MUC1 transgenic mice immunized with the IL-12-secreting cells.

Key Words

Breast Cancer, IL-12, Immunotherapy, Mucin, Transgenic mice.

INTRODUCTION

The immunogenic properties of highly malignant cells can be enhanced if the cells are genetically modified to secrete immune-augmenting cytokines. Neoplastic cells modified to secrete IL-2 (1-5), IL-4 (6,7), GM-CSF (8,9), interferon-gamma (IFN-g) (10, 11) or IL-12 (12,13) among others (14) are rejected by histocompatible mice. Under analogous circumstances, progressive tumor growth occurs in mice injected with unmodified tumor cells. The growth of the tumor leads, eventually, to the animals' death. The immunity in mice rejecting the cytokine-secreting cells, mediated primarily by cellular immune mechanisms, is directed toward unique tumor associated antigens expressed by both the cytokine-secreting as well as non secreting tumor cells. For this reason, cytokine-secreting tumor cells are under evaluation as potential immunotherapeutic agents (15-17).

Like other types of malignant cells, human breast cancer cells form unique antigens. Under appropriate circumstances, the antigens can become the targets of immune-mediated attack. One such antigen, PEM, has been extensively characterized and has been identified as a breast cancer associated T cell epitope (17). PEM is the product of the MUC1 gene. Antibody-mediated (18) and both major histocompatibility complex (MHC)-unrestricted (19, 20) as well as MHC-restricted class I cytotoxic T lymphocyte (CTL) responses (21) directed toward mucin have been identified in patients bearing breast neoplasms that express MUC1.

The MUC1 gene specifies a type I membrane glycoprotein that is naturally expressed on the apical surfaces of most glandular epithelial cells including the ductal epithelium of the breast. In contrast to mucin formed by non neoplastic cells, mucin formed by breast cancer cells is aberrant; it is underglycosylated and therefore differs antigenically from mucin expressed by non neoplastic cells (22, 23). Breast cancer associated mucin is antigenically weak, in the sense that mucin-positive breast neoplasms proliferate without apparent inhibition in breast cancer patients. Thus, the development of techniques that can successfully increase the antigenic properties of mucin-expressing breast carcinomas could be of importance in the treatment of patients with the disease.

Here, we took advantage of the development of transgenic mice that have been genetically modified to express human MUC1. The mice provide a unique opportunity to investigate the effect of cytokine-secretion by breast cancer cells modified to express human MUC1 on the cells' immunogenic properties. MUC1 transgenic mice express human MUC1 on glandular epithelial cells that produce mucin and are naturally tolerant to the molecule. The profile of expression and tissue distribution of MUC1 in MUC1 transgenic mice as well as the differences in the glycosylation pattern seen between normal breast epithelial cells and the malignant breast cancer cells are analogous to those found in humans (24, 25).

To determine if cytokine-secretion affected the immunogenic properties of breast cancer cells in MUC1 transgenic mice, a mouse breast cancer cell line (410.4) was first modified to express human MUC1 (410.4 cells that express human MUC1 are designated as E3 cells.) The cells were

then further modified to secrete IL-2, IL-4, interferon-g (IFN-g) or IL-12. The immunogenic properties of the cytokine-secreting cells were then tested in MUC-1 transgenic mice. The results indicated that unlike the other cytokines tested, immunization of the mice with E3 cells modified to secrete IL-12 resulted in generalized, long-term immunity toward the breast cancer cells and prolongation of survival of mice with breast cancer.

MATERIALS AND METHODS

Mouse mammary carcinoma cell lines

A mouse mammary carcinoma cell line, 410.4, originally isolated from a single, spontaneous mammary tumor that arose in a BALB/c mouse that was cross-fostered on an MMTV-carrying C3H mouse (26) was obtained from Bonnie Miller (Michigan Cancer Foundation, Detroit, MI). The cells were maintained under standard cell culture conditions (37^0C in a humidified 7% CO_2/air atmosphere) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS; Sigma, St Louis, MO), 1 mM sodium pyruvate, 100 units/ml penicillin and 100 ug/ml streptomycin (Gibco/BRL, Grand Island, NY) (growth medium). The cells were modified to express human MUC1, as described previously (27). In brief, 410.4 cells were co transfected (calcium phosphate co precipitation) with plasmid DNA specifying MUC1 (R1-MUC1-pEMSVscribe) and PY3, a plasmid specifying the hygromycin resistance gene (28). Colonies of hygromycin-resistant transfected cells were pooled and used in the experiments described below. The cells were maintained in growth medium under standard cell culture conditions. B16 cells, a melanoma cell line was kindly provided by I. Fidler (MD Anderson, Houston). Like 410.4 cells, it was maintained as a cell line under standard conditions.

Experimental animals

Six to eight week old BALB/c mice were from Charles River Laboratory (Wilmington MA, USA). Transgenic mice homozygous for the MUC1 gene and designated Sac II are H-2^k. Southern blotting using a human probe

that corresponded to approximately 500 base pairs of the tandem amino acid repeat domain of MUC1 was used to identify transgenic mice that had integrated the transferred human DNA sequence. The transgenic mice were then crossed with Balb/c mice to produce F_1 hybrids ($H\text{-}2^{k/d}$) to enable them to accept 410.4 cells and E3 cells (both are $H\text{-}2^{d}$).

All animals in the experiments were housed in our animal maintenance facility in accordance with the NIH guide for the Care and Use of Laboratory Animals.

Modification of E3 cells to form IL-12, IL-2, IL-4, or IFN-g.

A retroviral vector (TFG-mIL-12) (29), kindly provided by H. Tahara, (University of Pittsburgh, Pittsburgh, PA) was used to modify E3 cells to secrete IL-12. The vector specified the murine heterodimeric subunits (p40 and p35) of IL-12, and a gene conferring neomycin resistance. Both were under control of the TFG vector 5' LTR. E3 cells were transduced with TFG-mIL-12, according to the protocol described by Tahara et al., (29). In brief, 5 X 10^5 cells in growth medium were added to individual wells of a 6 well plate (Falcon). Twenty four hours later, 1 ml of TFG-mIL-12-Neo retroviral supernatant in the presence of polybrene (8 ug/ml) was added to each well. After 24 hours further incubation, the cells were washed and the medium was replaced with growth medium containing 400 ug/ml of the neomycin analog, G418. (One hundred percent of non transduced cells die in growth medium containing 400 ugm G418.) After a 14 day period of incubation, cells proliferating in the G418-containing medium were pooled and maintained as a cell line. As a control, the same protocol was followed except that a plasmid (pZipNeoSV-X, from M.K.L.

Collins, University College, London, England) was substituted for TFG-mIL-12. (pZipNeoSV-X specifies a neomycin resistance gene, but not the gene for a cytokine.) Lipofectin™ (Gibco/BRL, Grand Island, New York) was used to facilitate uptake of pZipNeoSV-X by E3 cells, according to the manufacturer's instructions (Gibco/BRL, Grand island, NY).

A similar procedure was followed to modify E3 cells for the secretion of IL-2, IL-4 or IFN-g. Eukaryote expression vectors pZipNeoSV-IL-2, pZipNeoSV-IL-4 and pZipNeoSV-IFN-g (obtained from M.K.L. Collins, University College, London) were used for this purpose. pZipNeoSV-IL-2, pZipNeoSV-IL-4 and pZipNeoSV-IFN-g encode human IL-2, mouse IL-4 and mouse IFN-g respectively and a gene conferring resistance to the neomycin analog, G418. In brief, 1 X 10^6 E3 cells were seeded into 100 mm cell culture dishes in 10 ml of growth medium. After incubation for 18 hrs., the cells were washed with DMEM (Gibco/BRL) followed by the addition of 10 ug of the vector in 100 ul DMEM and 100 ul of Lipofectin, prepared according to the manufacturer's instructions (Gibco/BRL). After 24 hrs. further incubation, the cells were washed and the medium was replaced with growth medium containing 400 ug/ml G418 (Gibco/BRL). Colonies of cells proliferating in G418-containing growth medium were pooled and maintained as cell lines. Every third passage, the transfected cells were cultured in growth medium containing 400 ug/ml G418.

Detection of cytokine formation by retrovirally transduced E3 cells.

The formation of IL-12, IL-2, IL-4, or IFN-g by the transduced cells was detected by ELISA. In brief, 10^6 cells transduced with the relevant vector were added to cell culture flasks containing 10 ml of RPMI medium

(Gibco/BRL) supplemented with 2.0 mM Na pyruvate, 10 percent FCS (Sigma), 0.15 M Hepes and antibiotics. After incubation for 48 hrs., the cell culture supernatants were assayed for the presence of the relevant cytokines, following the directions of the supplier (Endogen, Woburn, MA).

Immunofluorescent staining and cytofluorometric measurements.

Quantitative immunofluorescent measurements were used to detect the expression of human MUC1 by E3 cells transduced with pZipNeoSV-IL-2, pZipNeoSV-4, pZipNeoSV-IFN-g or TFG-mIL-12. The measurements were performed in a FACS Caliber, (Becton-Dickinson). For the analysis, a single cell suspension was prepared from the monolayer cultures of the retro-virally transduced cells using 0.1 mM EDTA in 0.1 M phosphate buffered saline (pH 7.4 (PBS). The cells were washed with growth medium containing 0.2% sodium azide and 0.5% FCS. Afterward, a monoclonal antibody (SM-3) that reacts with mucin expressed by human breast cancer cells (30) was added to the cells, followed by incubation at 4^0 for 1 hr. After incubation, the cells were washed with PBS containing 0.2% sodium azide and 0.5% FCS, followed by the addition of rabbit anti mouse immunoglobulin antibody conjugated to FITC (Sigma, St. Louis) and further incubation at 4^0 for 1 hr. After further washing, the cells were analyzed by quantitative immunofluorescent staining. One parameter fluorescence histograms were generated by analyzing 1 X 10^4 cells. Background staining was determined by substituting cells stained with rabbit anti mouse immunoglobulin antibody conjugated to FITC alone.

Measurement of the growth of E3 cells modified for cytokine-secretion in MUC1 transgenic mice.

Two dimensional measurements were used to determine the growth of E3 cells modified for cytokine-secretion that were injected into breast tissues of MUC1 transgenic mice. A vernier caliper was used for this purpose. The volume of the tumor was calculated by the formula $0.4ab^2$, where a = length and b = width of the tumor.

Results

1. MUC1 transgenic mice express human MUC1.

Immunohistochemical staining for PEM specified by the human MUC1 gene was used to determine if the MUC1 transgenic mice used in the experiments expressed human PEM. Selected organs of the mice were tested, using a monoclonal antibody (HMFG-1) found previously to react with the amino acid sequence Pro-Asp-Thr-Arg-Pro of the core protein of human PEM. The results (Fig 1), indicate that PEM was expressed on the apical surfaces of cells in the ductal epithelium of distal convoluted tubules of the kidney, epithelial cells lining bronchioles of the lung and bile duct epithelium of cells in the liver. Under similar conditions, sections of the same tissues from transgenic mice incubated with the secondary antibody alone failed to stain.

2. 410.4, a mouse breast cancer cell line, transduced with R1-MUC1-pEMSVscribe expresses human MUC1.

The protein core of mucin formed by human breast cancer cells specifies the immunodominant epitope (30, 31). It is "exposed" since mucin is aberrantly expressed by mucin producing epithelial carcinoma cells. A mouse breast cancer cell line (410.4) that originated in a BALB/c mouse ($H\text{-}2^d$) (26) was modified to express human mucin by transduction with R1-MUC1-pEMSVscribe, a vector that specified the coding sequence. Quantitative immunofluorescent staining was used to determine if MUC1 was expressed by the transduced cells.

Either of two monoclonal antibodies, SM3 and HFMG-1 (like HFMG-1, SM3 is specific for the amino acid sequence Pro-Asp-Thr-Arg-Pro of the core protein) were used in the study. As indicated (Figure 2) the mean fluorescent index (MFI) of the transduced cells incubated with SM3 antibodies, followed by incubation with FITC-conjugated rabbit anti mouse immunoglobulin (Ig) (Sigma) was significantly ($P < .01$) higher than the MFI of cells incubated with FITC conjugated rabbit anti mouse immunoglobulin alone, taken as "background." The MFI of non transduced 410.4 cells stained with SM3 antibodies was not above this "background" (Fig 2). The MFI of transduced cells stained with HFMG-1 antibodies was equivalent to that of the cells stained with SM3 antibodies (Figure 2).

3. E3 cells express low levels of MHC class I-determinants.

Neoplastic cells may express low levels of MHC class I-determinants, a documented means of tumor cell "escape" from immune-mediated destruction (reviewed in 33). Quantitative immunofluorescent measurements were performed, using FITC-labeled H-2K^d antibodies (Pharmingen), to determine the level H-2K^d class I determinants expressed by E3 cells. For comparison, the level of H-2K^d determinants expressed by non neoplastic nucleated spleen cells from MUC1 transgenic mice was determined as well. The results indicated that like other types of cancer cells, E3 cells formed lesser quantities of class I-determinants than non neoplastic cells from MUC1 transgenic mice. The MFI of E3 cells stained with H-2K^d antibodies was significantly less than that of nucleated spleen cells from the mice (86.4. and 281 respectively, ($p < .01$).

As controls, E3 cells were stained with FITC-labeled H-2K^k antibodies (410.4 cells of BALB/c origin are not expected to form H-2K^k-determinants) or with isotype control serum, both followed by FITC rabbit anti mouse Ig. The MFI of E3 cells stained with FITC-labeled H-2K^k antibodies was not significantly different than that of E3 cells stained with the isotype control serum alone.

4. E3 cells formed slowly growing tumors in immunocompetent BALB/c mice.

Human MUC1 is immunologically foreign in (non transgenic) BALB/c mice. To determine if the molecule was sufficiently antigenic to inhibit the cells' tumorigenic properties, that is, to lead to rejection of the tumor cells, immunocompetent BALB/c mice were injected into the fat pad of the breast with 1 X 10^6 E3 cells, or for comparison, with an equivalent number of (MUC1-negative) 410.4 cells. The animals were monitored for the time to first appearance of tumor (the latent period) and the rate of tumor growth at the injection sites. The results (Fig 3) indicated that progressively growing breast neoplasms formed at the injection sites in both instances. However, the latent period was significantly prolonged in the mice injected with E3 cells relative to that of BALB/c mice injected with 410.4 cells (50 +/- 12 days versus 16 +/- 1 day respectively, $P < .01$). Once the tumors appeared, however, the rate of tumor growth in mice injected with E3 cells was not significantly different than the rate of tumor growth in mice injected with 410.4 cells.

Cells recovered from the injection sites were re established as breast cancer cell lines. An analysis by ELISA of the culture supernatants was performed to determine if the cells were producing cytokines. The results (not presented) indicated that the cells were producing equivalent quantities of the same cytokine as the cells first injected.

Conceivably, the generation time of E3 cells was longer than the generation time of 410.4 cells and this difference was responsible for the delayed appearance of the tumor of E3 cells in the BALB/c mice. This question was investigated by comparing the proliferation rates of E3 cells and 410.4 breast cancer cells in vitro. The results indicated that the generation times (approximately 24 hours in each instance) of the two cell types were not significantly different from each other. As noted previously, the rates of tumor growth in mice injected with E3 cells or 410.4 cells were not significantly different from each other.

5. E3 cells formed progressively growing tumors in MUC1 transgenic mice.

MUC1 transgenic mice express human mucin and would be expected to be naturally tolerant to histocompatible cells that express human MUC1. To determine if E3 cells formed tumors in the transgenic mice, 1 X 10^6 viable E3 cells were injected into the fat pad of the breast of MUC1 transgenic mice and the latent period and the rate of tumor growth were compared with the latent period and rate of tumor growth in MUC1 transgenic mice injected with an equivalent number of 410.4 cells. As indicated (Fig 4), unlike the injections in BALB/c mice, the latent period

and rate of tumor growth in MUC1 transgenic mice injected with E3 cells were not significantly different that the latent period and rate of tumor growth in transgenic mice injected with 410.4 cells. The mice exhibited no resistance to the growth of breast cancer cells modified to express human MUC1.

6. Cytokine secretion by E3 cells transduced with retroviral vectors specifying cytokine genes.

Cytokine-secretion by cancer cells augments the cells' immunogenic properties (1-14). Several immune augmenting cytokines were evaluated to determine if cytokine-secretion by E3 cells affected the cells' immunogenic properties in MUC1 transgenic mice. As a first step, the cells were modified to secrete IL-2, IL-4, IFN-g or IL-12. Retroviral vectors encoding the relevant cytokine gene and a gene conferring resistance to the neomycin analog, G418, were used for this purpose. As a control, E3 cells were transduced with a vector (pZipNeoSV-X) which specified the neomycin resistance gene but did not encode a cytokine gene.

After selection in growth medium containing sufficient quantities (400 ug/ml) of G418 to kill one hundred percent of non transduced E3 cells, the antibiotic-resistant cells were maintained as cell lines. After 48 hrs. incubation, culture supernatants from the cells were analyzed by ELISA for the presence of the relevant cytokine. The results indicated that 10^6 cells transduced with a vector encoding the gene for IL-2 (pZipNeoSV-IL-2) formed 72 units/ml IL-2, cells transduced with a vector encoding the

gene for IL-4 (pZipNeoSV-IL-4) formed 5 pg/ml IL-4, cells transduced with a vector encoding the gene for interferon gamma (IFN-g) (pZipNeoSV-IFN-g) formed 1.2 ng/ml IFN-g and cells transduced with a vector encoding the gene for IL-12 (TFG-mIL-12) formed 2.54 ng/ml IL-12. Under similar conditions, the culture supernatants of non transduced E3 cells, or E3 cells transduced with pZipNeoSV-X failed to contain detectable quantities of IL-2, IL-4, IFN-g or IL-12. Every third passage, the cytokine-secreting cells were placed in growth medium containing 400 ug/ml G418. Under these circumstances, equivalent quantities of the relevant cytokines were detected when the cells were reanalyzed after three months of continuous culture (these data are not presented).

7. IL-12 secretion by E3 cells inhibited the cells' tumorigenic properties in MUC1 transgenic mice.

To determine if cytokine-secretion by E3 cells affected their tumorigenic properties, MUC1 transgenic mice were injected into the fat pad of the breast with 1 X 10^6 E3 cells modified to secrete IL-2, IL-4, IFN-g or IL-12. For comparison, the mice were injected with an equivalent number of non cytokine-secreting E3 cells transduced with the vector (pZipNeoSV(X) that conferred neomycin resistance but did not specify a cytokine gene. As indicated (Figure 5), the latent period was prolonged in the group of MUC1 transgenic mice injected with IL-12-secreting E3 cells (E3-IL-12 cells), relative to that of any of the other groups ($P < .01$). Six of the 8 mice injected with E3-IL-12 cells failed to form tumors and appeared to have rejected the breast cancer cells. Two mice injected with E3-IL-12 cells formed slowly growing tumors at the injection sites that led

eventually to the animals' death. The median survival time (MST) of these animals, approximately 100 days, was significantly ($p < .001$) longer than the MST of mice in any of the other groups (Figure 6). H and E staining of tissue sections taken from the sites of injection of mice injected with E3-IL-12 cells revealed an intense inflammatory infiltrate consistent with the rejection of the cytokine-secreting cells (Figure 1, panels a, b and c).

The latent period in MUC1 transgenic mice injected with E3 cells modified to secrete IFN-g was found to be less than that of any of the other groups (Fig 6), suggesting that modification of the cells to secrete IFN-g augmented the cells' tumorigenic properties. This point was not investigated further, but is consistent with the report of Puisieux et al., who noted a similar phenomenon (34).

8. Immunity to breast cancer developed in MUC1 transgenic mice that rejected E3 cells modified to secrete IL-12.

Tumors failed to form in the majority of MUC1 transgenic mice injected with IL-12-secreting E3 cells. To determine if the mice that rejected the IL-12-secreting cells developed immunity to E3 cells, that is, if they were resistant to the growth of (non cytokine-secreting) E3 cells, the surviving mice received a challenging injection of E3 cells 71 days after the injection of E3-IL-12 cells. As indicated (Fig 6) none of the animals formed tumors. Under similar conditions, one hundred percent of naive MUC1 transgenic mice injected with non cytokine-secreting E3 cells

developed progressively growing neoplasms at the injection sites that led to the animals' death.

9. Immunity to E3 cells in transgenic mice injected with E3-IL-12 cells.

Inhibition of the growth E3 cells in the MUC1 transgenic mice that rejected E3-IL-12 cells suggested that the resistance was mediated by immune mechanisms. An immunoassay that uses cytokine-release by antigen-stimulated spleen and lymph node cells as an indication of an immune response (35) was used to investigate this question. In the experiment, naive MUC1 transgenic mice received a single subcutaneous injection of 1 X 10^6 E3-IL-12 cells. Two weeks later, the mice were sacrificed and pooled cell suspensions were prepared from the spleens and regional lymph nodes. The cell suspensions were co incubated for 24 hrs under standard cell culture conditions with X-irradiated (5000 rads from a ^{60}Co source) E3 cells or, for comparison, with X-irradiated 410.4 cells, or with X-irradiated B16 cells, a melanoma cell line, used as a specificity control. As an additional control the spleen/lymph node cell suspensions were incubated under the same conditions without the addition of the X-irradiated cells. After incubation, the culture supernatants were analyzed by ELISA for the presence of IFN-g. The results (Table 1) indicated that the titers of IFN-g in the culture supernatants of cells from the immunized mice co-incubated with X-irradiated E3 cells or X-irradiated 410.4 cells were significantly higher ($P < .01$) than the titers of IFN-g in the culture supernatants of cells co-incubated with X-irradiated B16 cells. IFN-g was undetectable in the supernatants of spleen/lymph node cell cultures that were incubated

without the addition of X-irradiated tumor cells. Analogous findings were observed if the culture supernatants were analyzed for the presence of MIP1a, a chemokine (36). The titers of MIP1a in culture supernatants of spleen/lymph node cell suspensions from mice immunized with E3-IL-12 cells co-incubated with X-irradiated E3 cells or X-irradiated 410.4 cells were significantly higher ($P < .01$) than present in culture supernatants from cell suspensions co-incubated with X-irradiated B16 cells, or cell suspensions incubated alone (Table 1). These results indicated that immunization with E3-IL-12 cells specifically enhanced the immune response toward both E3 cells and 410.4 cells.

An immunofluoresence assay was used to determine if antibodies reactive with E3 cells were present in the sera of MUC1 transgenic mice immunized with E3-IL-12 cells. Thirty days after a single injection of 1 X 10^6 E3-IL-12 cells, pooled sera from the mice were incubated with E3 cells, followed by further incubation with FITC-conjugated anti mouse IgG, IgM or IgA to determine the isotype of the antibodies that reacted with E3 cells. The results (Table 2) indicated that E3 cells incubated with sera from mice immunized with E3-IL-12 cells followed by FITC-conjugated anti mouse IgG reacted positively. Under similar conditions, E3 cells incubated with sera from mice immunized with E3-IL-12 cells followed by anti mouse IgM or FITC-conjugated anti mouse IgA failed to react. Thus, IgG was the major immunoglobulin class reactive with E3 cells in mice immunized with E3-IL-12 cells. The requirement for IL-12-secretion by E3 cells in the generation of the antibody response was supported by the finding that immunization of MUC1 transgenic mice with (non cytokine-secreting) E3 cells or (non cytokine-secreting) 410.4 cells

failed to generate an antibody response toward the breast cancer cells (Table 2).

DISCUSSION

Under ordinary circumstances, inbred mice injected with histocompatible breast cancer cells develop progressively growing neoplasms that lead, eventually, to the animal's death. The malignant cells do not provoke effective anti tumor immune responses as the duration of survival can be (inversely) related to the number of cancer cells injected.

The studies reported here were prompted by the finding that human PEM, the product of the MUC1 gene, is expressed in an altered form by breast cancer cells is a breast cancer antigen. Under appropriate circumstances, breast cancer cells that express PEM can be recognized by cytotoxic T lymphocytes and can become targets of immune-mediated attack. Clinical studies are in progress to test the immunotherapeutic benefits of tumor vaccines that express PEM (15-17).

MUC1, the gene for PEM, codes for a heavily glycosylated macromolecule found on the surface membranes of both normal and malignant mucin-producing epithelial cells. The glycosylation pattern of PEM expressed by breast cancer cells can be distinguished from the glycosylation pattern of PEM expressed by non malignant cells of the same individual. Mucin formed by breast cancer cells is over expressed and underglycosylated and differs antigenically from mucin expressed naturally by non malignant cells epithelial cells of the breast. As a consequence, novel T cell epitopes are exposed that are potentially antigenic. The underglycosylated PEM can be a target for both major histocompatibility complex (MHC)-unrestricted as well as MHC-restricted class I cytotoxic

T lymphocyte (CTL) responses. T cell mediated cytotoxicity responses toward mucin have been identified in patients bearing breast neoplasms that express MUC1. However, the natural antigenic properties of PEM are insufficient to stimulate immune responses that are capable of leading to tumor cell rejection.

The long-term objective of our work is the investigation of various means of increasing the immunogenic properties of mucin expressed by breast cancer cells, with a view towards developing an immunotherapeutic agent that can be used in the treatment of breast cancer patients. The successful development of MUC1 transgenic mice that express human mucin enables the study to be carried out in experimental animals. Like breast cancer patients, the mice are naturally tolerant to human mucin. As other cellular constituents, mucin is expressed most strongly at the apical surfaces of ductal epithelial cells of the lactating breast (37), and is viewed as "self" by the animal's immune system. The injection of a highly malignant mouse breast cancer line modified to express human MUC1 into the breast of MUC1 transgenic mice mimics as closely as possible mucin-producing breast cancer in patients.

Here, the MUC1 gene was introduced into 410.4 cells, a highly malignant breast cancer cell line of BALB/c origin (H-2^d) and the cells (E3) were tested for their immunogenic properties in MUC1 transgenic mice. The validity of the model was emphasized by the finding that the latent period and rate of tumor growth growth of E3 cells in MUC1 transgenic

mice were essentially the same as the latent period and rate of tumor growth of 410.4 cells.

The mouse breast cancer cells modified to express human MUC1 were further modified to produce various cytokines known to augment the immunogenic properties of malignant cells. We hypothesized that presentation of MUC1 to the immune system in the microenvironment of immune-augmenting cytokines would generate an immune response to MUC1 expressed by the breast cancer cells.

The results clearly indicated that modification of the MUC-1-expressing breast cancer cells to secrete IL-12 increased the cells' immunogenic properties in the MUC1 transgenic mice. Six of 8 mice injected with the IL-12-secreting cells failed to form tumors and appeared to have rejected the IL-12-secreting cells. (The remaining two mice that eventually formed tumors did so after a prolonged latent period.) When tested at a later time, the animals were completely resistant to (non cytokine-secreting) MUC1-positive breast cancer cells. They developed cellular immune responses toward the cells, as indicated by studies performed in vitro, and the presence of an intense inflammatory infiltrate at the site of injection of the IL-12-secreting breast cancer cells. IgG antibodies reactive with the cells developed in MUC1 transgenic mice injected with E3-IL-12 cells. Whether or not the antibodies play a role in eradication of the tumor cells was not determined.

IL-12 is a pleotropic, heterodimeric immune-augmenting cytokine that stimulates both natural killer (NK) and T lymphocytes to produce interferon-gamma and tumor necrosis factor-alpha. It also promotes the development of Th1 $CD4^+$ cells, which are also involved in the induction of cellular immunity (38-39). It also enhances the activity of tumor infiltrating lymphocytes. IL-12 has been previously described as a strong immune-augmenting cytokine (40-42). Our results extend these studies to a mouse model of breast cancer in patients.

The potential importance of these findings to the treatment of breast cancer patients is indicated by reports indicating that spontaneous breast neoplasms arising in patients, like other types of cancer, are potentially immunogenic. The malignant cells form various tumor associated antigens such as HER-2/neu (43, 44), mutant p53 (45) MAGE-1 (46), BAGE (47) as well as the protein core of mucin that can be recognized by cytotoxic T lymphocytes. These may be only several examples of an array of breast cancer associated antigens that arise from altered genes in the malignant cells. Genetic instability is a common characteristic of breast cancer and other types of neoplastic cells (48-50).

In an experimental system that mimics breast cancer in patients, the data presented here indicate that in the immunogenic properties a mucin-expressing adenocarcinoma of the breast can be enhanced if the cells are modified to secrete IL-12. They point toward the possible immunotherapeutic potential of breast cancer cells modified for IL-12-

secretion. Our findings may be of importance in the clinical care of breast cancer patients.

Acknowledgment

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Figure 1.

Immunohistochemical and H and E staining of various tissues of MUC1 transgenic mice stained with an antibody (HFMG-1) reactive with human MUC1.

Legend:

(a) = Inflammatory infiltrate at the site of injection of E3-IL-12 cells into breast tissue of MUC1 transgenic mice. H and E section (250X)

(b) = Inflammatory infiltrate at the site of injection of E3-IL-12 cells into breast tissue of MUC1 transgenic mice. H and E section (400X)

(c) = Inflammatory infiltrate and a mucin positive breast cancer cell (arrow) in breast tissue from MUC1 transgenic mice injected with E3-IL-12 cells. Immunohistochemical staining (400X)

(d) = Immunohistochemical staining of portal tracts in the liver of MUC1 transgenic mice with immunostaining localized to bile duct epithelium (400X)

(e) = Immunohistochemical staining of portal tracts in the liver of MUC1 transgenic mice, showing strong luminal surface staining (1000X)

(f) = Immunohistochemical staining of bronchial epithelium of MUC1 transgenic mice (1000X)

(g) = Immunohistochemical staining of bronchial epithelium of MUC1 transgenic mice (400X)

(h) = Immunohistochemical staining of renal cortex of MUC1 transgenic showing reaction with cells in the distal convoluted tubules (400X)

(i) = Immunohistochemical staining of renal cortex of MUC1 transgenic mice showing reaction with cells in the distal convoluted tubules (1000X)

For the immunohistochemical staining, the tissues were fixed in methanol:chloroform:acetic acid, (60:30:10) and paraffin embedded for sectioning. Dewaxed paraffin sections were incubated with 50% fetal calf serum in PBS for 30 min to prevent non specific binding of antibodies. The blocking solution was then removed and replaced by neat hybridoma culture supernatant, followed by incubation at room temperature for 60 min. After three 5 min washes with PBS, the sections were incubated for 1 hr. with peroxidase-conjugated rabbit anti mouse immunoglobulin antiserum (Dako; diluted 1:50 in PBS with 15% fetal calf serum). The slides were then washed three times with PBS. The substrate solution consisting of .03 percent hydrogen peroxide in PBS and 1 mg/ml diaminobenzidine (Sigma) was added and the reaction was allowed to continue for 5 to 8 min. After final washing with PBS, the slide were counterstained with H and E and mounted.

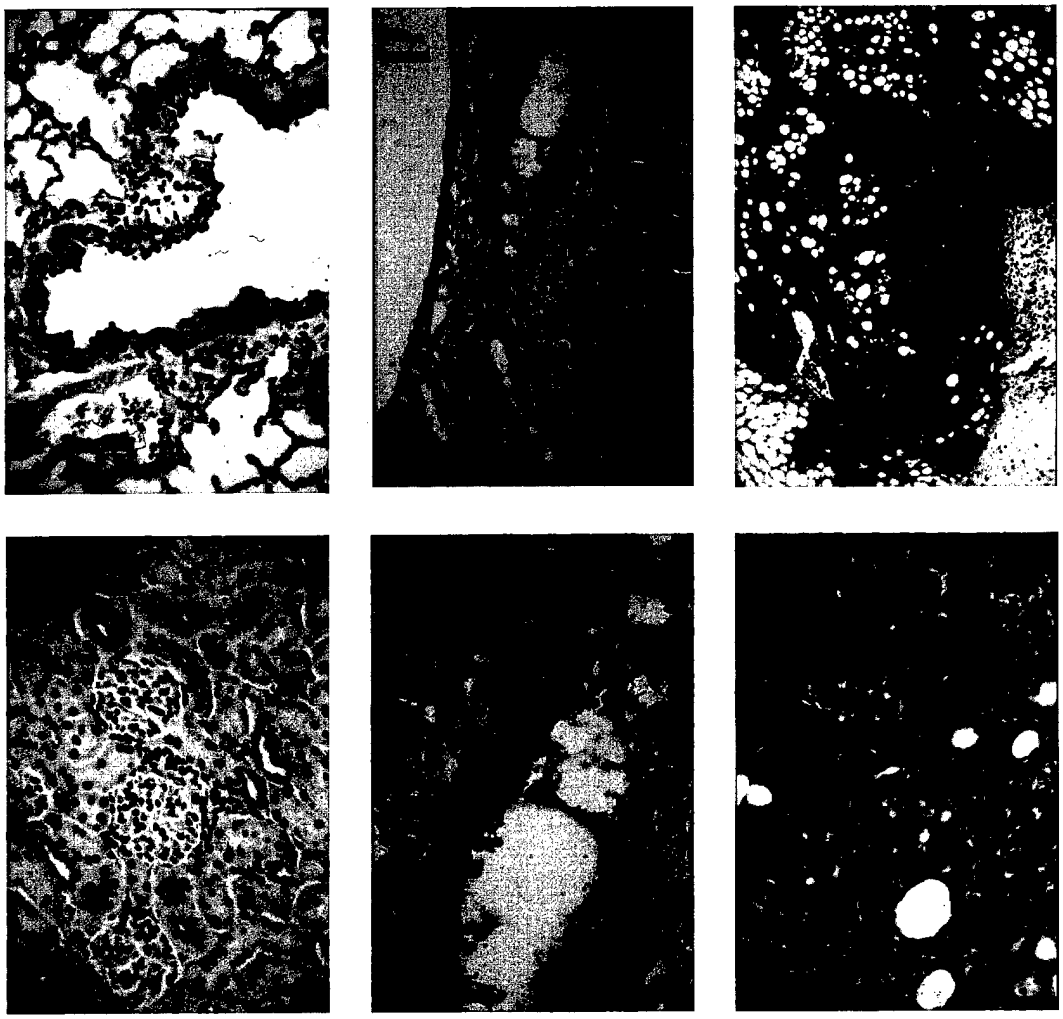
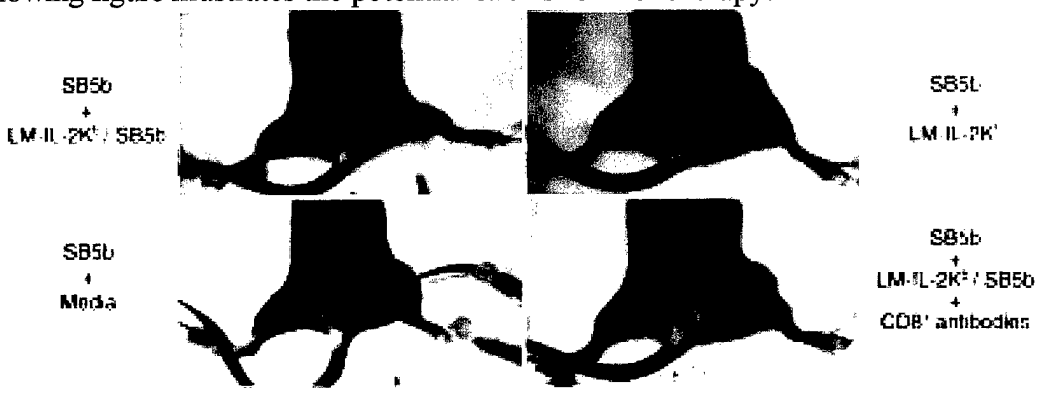


Figure 2

Tumor growth in C3H mice immunized with IL-2-secreting LM fibroblasts transfected with DNA from breast cancer cells followed by a challenge injection of breast cancer cells from a neoplasm arising in a C3H mouse

The following figure illustrates the potential of this form of therapy:



Legend: C3H mice received three s.c. injections at weekly intervals of 5 X 10^6 cells. One week after the last injection, the mice were injected with 5 X 10^3 breast cancer cells from a neoplasm arising in a C3H mouse (SB-5b). The photo was taken 35 days after the injection of the breast cancer cells. SB5b + LM-IL-2K^b/SB5b = Immunized with LM fibroblasts modified for IL-2-secretion and the expression of H-2K^b determinants transfected with DNA from SB5b, a breast neoplasm that arose spontaneously in a C3H mouse. SB5b + LM-IL-2K^b = Immunized with LM fibroblasts modified for IL-2-secretion and the expression of H-2K^b determinants but not transfected with DNA from the breast cancer cells. SB5b + media = Injected with SB5b cells alone. SB5b + LM-IL-2K^b/SB5b + CD8 antibodies = The mice were depleted of CD8 cells before immunization with LM-IL-2K^b/5b cells followed by the injection of the cancer cells.

Figure 3

FIGURE LEGEND

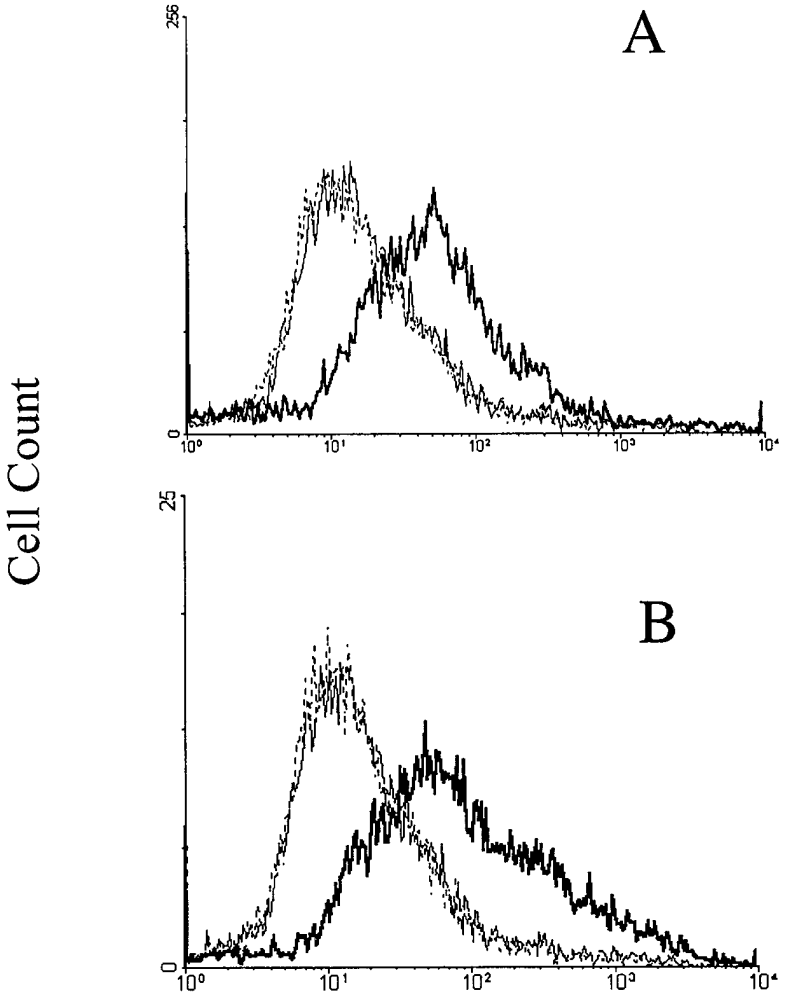
H and E Staining of Organs from C3H/He Mice Immunized with LM-IL-K^b/SB5b Cells

C3H/He female mice between 8 and 12 weeks of age were injected subcutaneously three times at weekly intervals with 5 X 10^6 LM fibroblasts modified to express H-$2K^b$-determinants and to secrete IL-2, that were transfected with DNA from a breast neoplasm that arose spontaneously in a C3H/He mouse (LM-IL-K^b/SB5b cells). One week after the last injection, the mice were sacrificed and tissue sections from various organs were stained with H and E.

(a) = adrenal
(b) = joint
(c) = kidney
(d) = liver
(e) = ovary
(f) = spleen.

None of the organs exhibited signs of inflammation or other abnormal findings.

e


A
B
256
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25
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10^0
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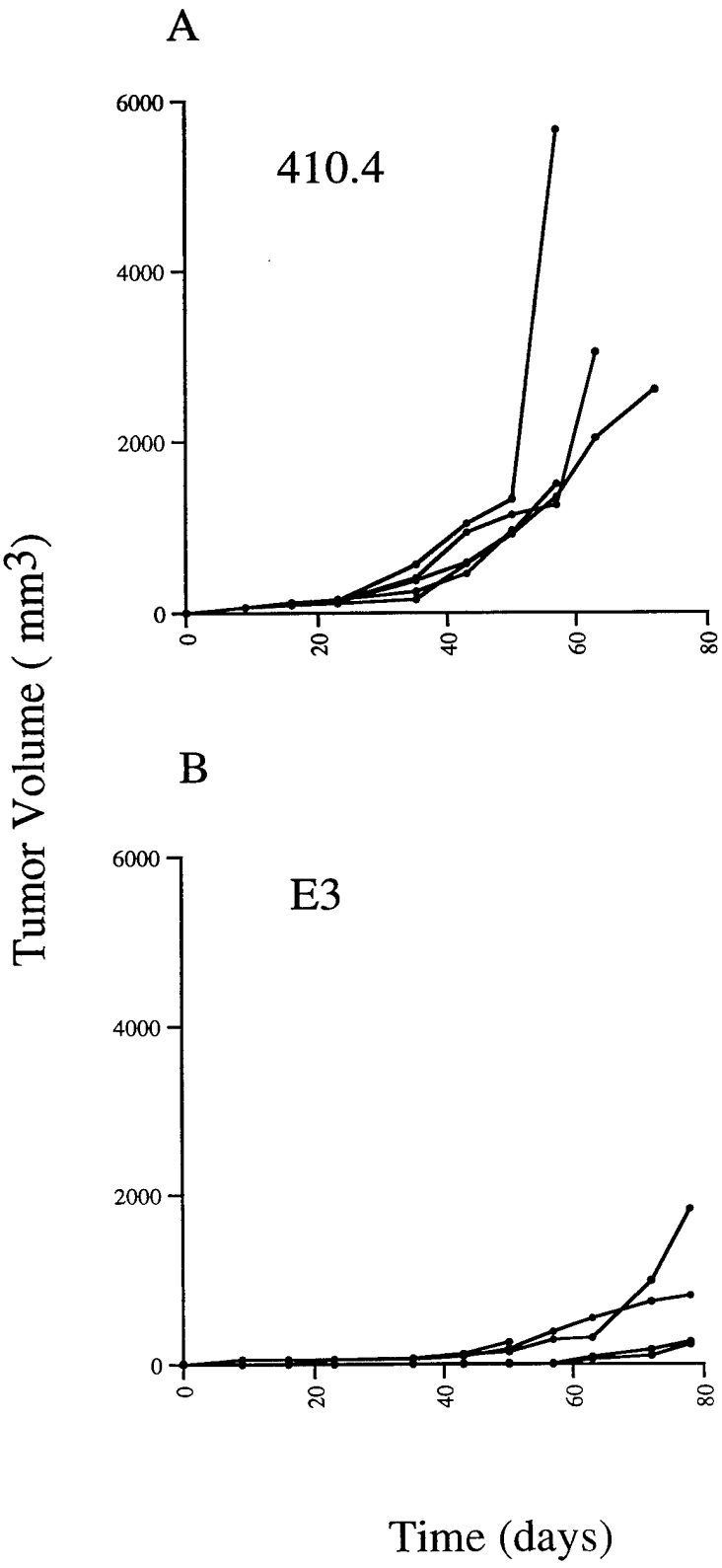
10^3
10^4
Cell Count
Flourescence Intensity


A
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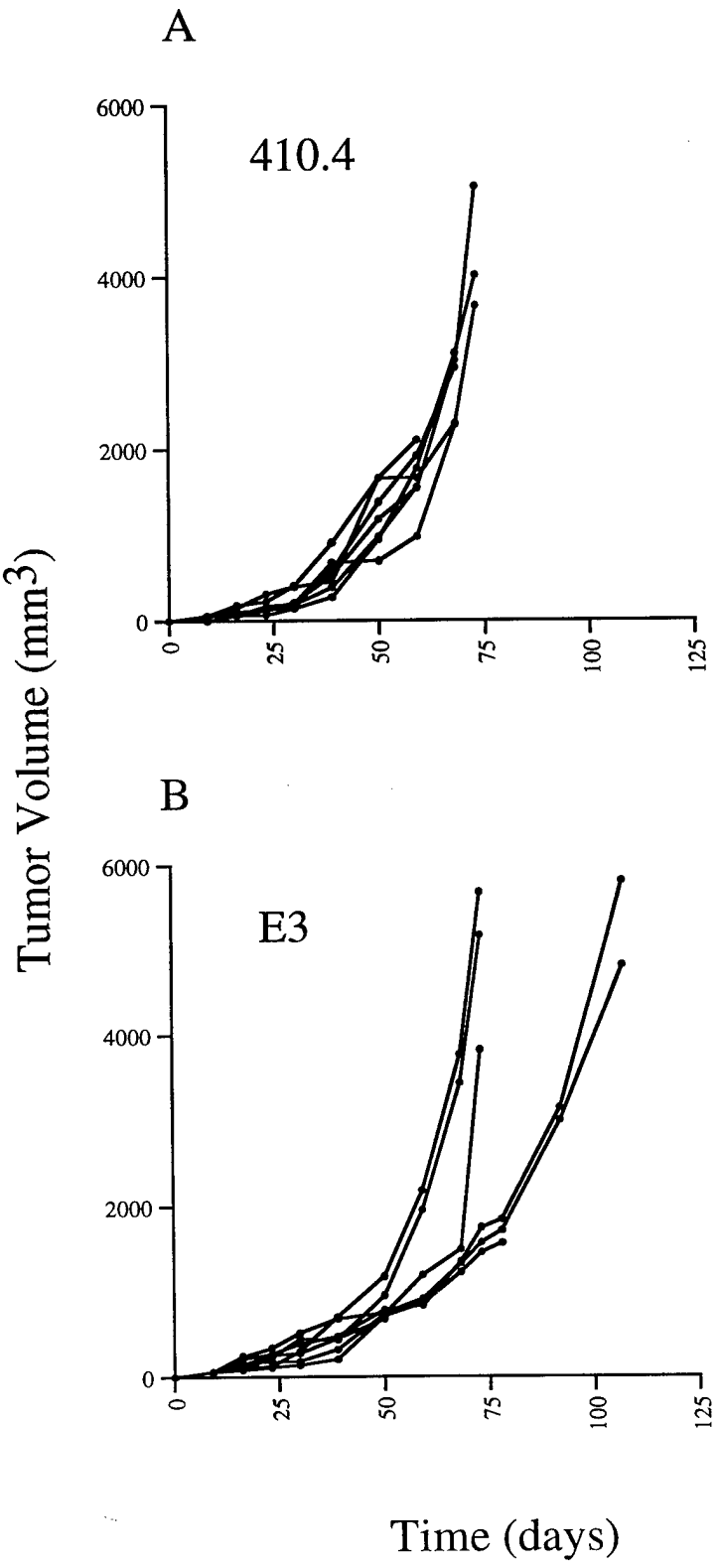
6000
4000
2000
0
0
20
40
60
80
B
E3
6000
4000
2000
0
0
20
40
60
80
Tumor Volume (mm3)
Time (days)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0
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125
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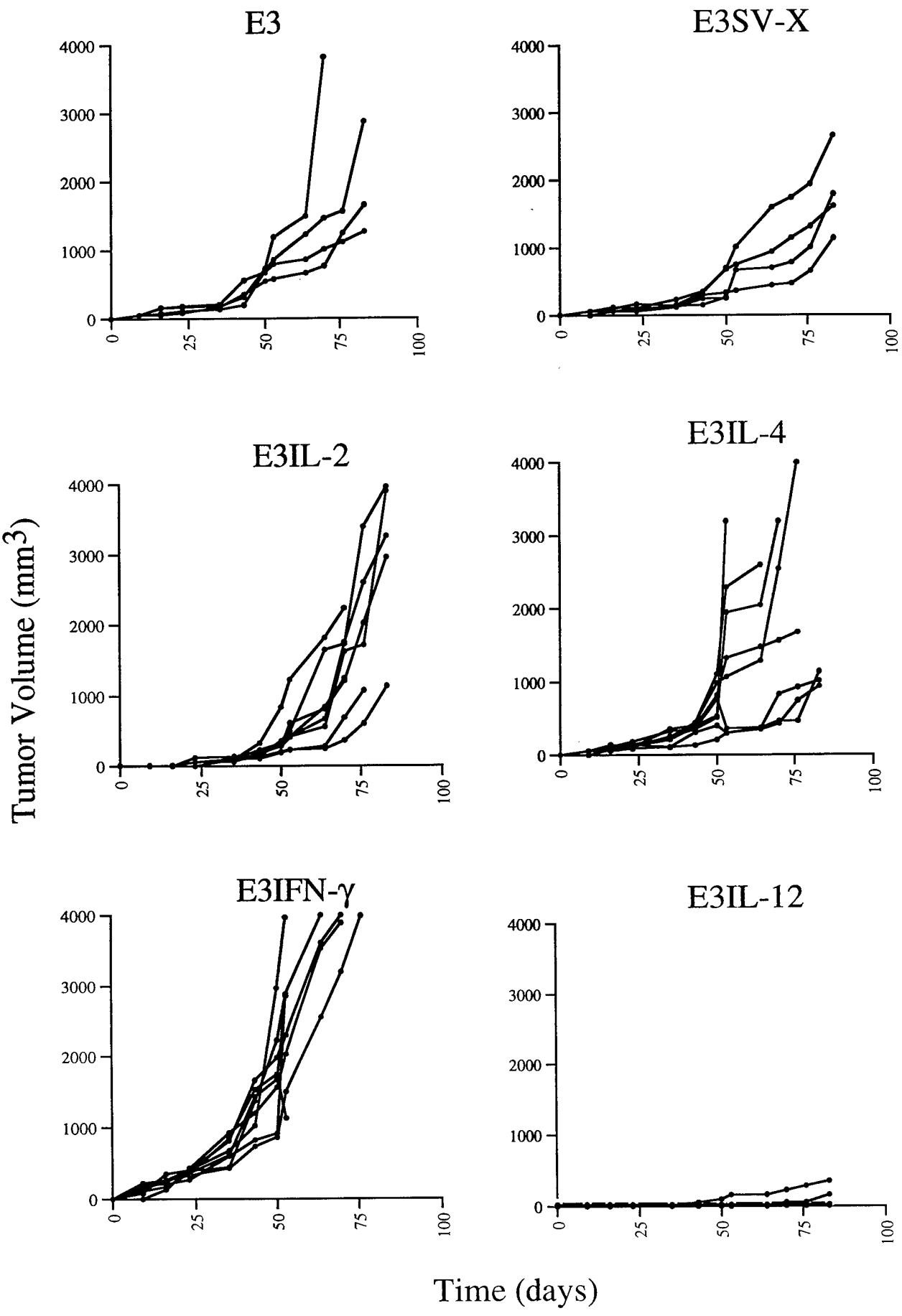
50
75
100
125
Tumor Volume (mm3)
Time (days)


E3
E3SV-X
E3IL-2
E3IL-4
E3IFN-γ
E3IL-12
Tumor Volume (mm3)
Time (days)
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2000
1000
0
0
25
50
75
100

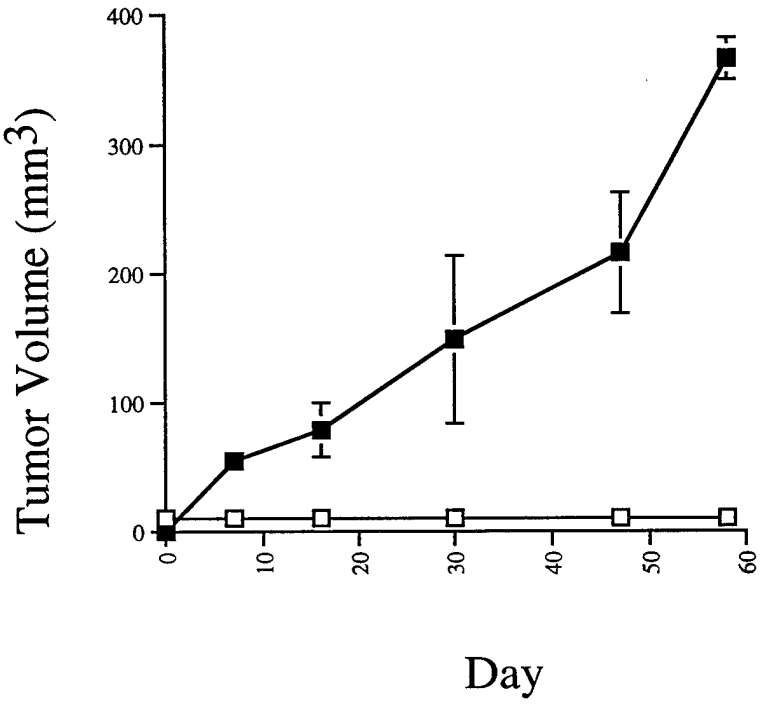
Tumor Volume (mm3)
400
300
200
100
0
0
10
20
30
40
50
60
Day

TABLE 1 Cytokine release from a suspension of spleen and lymph node cells from MUC-1 transgenic mice injected with E3-IL-12 cells co-incubated with irradiated E3 cells

	lymphocytes from mice primed with			
Cytokine (pg/ml)	E3	410.4	B16	no stim
IFN-γ	1416±94	998±44	101±4	0
MIP1-α	252±12	303±6	37±3	25±8

TABLE 2 Isotype of E3 antibodies in MUC-1 transgenic mice immunized with E3-IL-12 cells

Serum from mice immunized with	% Staining of E3 cells		
	IgG	IgM	IgA
E3IL12	72±10	10±3	0.3±0.2
E3	7±3	5±2	0
410.4	6±3	4±3	0

Treatment of Breast Cancer with Fibroblasts Transfected with DNA from Breast Cancer Cells[1]

Edwin de Zoeten,[2]* Victoria Carr-Brendel,[2]* Dubravka Markovic,*
Joyce Taylor-Papadimitriou,† and Edward P. Cohen[3]*

This investigation was based on the hypothesis that weakly immunogenic, breast cancer-associated Ags, the products of mutant or dysregulated genes in the malignant cells, will be expressed in a highly immunogenic form by semiallogeneic IL-2-secreting fibroblasts transfected with DNA from breast cancer cells. (Classic studies indicate that transfection of genomic DNA can stably alter both the genotype and the phenotype of the cells that take up the exogenous DNA.) To investigate this question, we transfected LM mouse fibroblasts (H-2^k) modified to secrete IL-2 with genomic DNA from a breast adenocarcinoma that arose spontaneously in a C3H/He mouse (H-2^k). To increase their nonspecific immunogenic properties, the fibroblasts were also modified before transfection to express allogeneic MHC determinants (H-2K^b). Afterward, the IL-2-secreting semiallogeneic cells were cotransfected with DNA from the spontaneous breast neoplasm, along with a plasmid (pHyg) conferring resistance to hygromycin. Pooled colonies of hygromycin-resistant cells were then tested in C3H/He mice for their immunotherapeutic properties against the growth of the breast neoplasm. The results indicated that tumor-bearing mice immunized with the transfected cells survived significantly longer than mice in various control groups. Similar beneficial effects were seen in C57BL/6 mice injected with a syngeneic breast carcinoma cell line (EO771) and semiallogeneic, IL-2-secreting fibroblasts transfected with DNA from EO771 cells. The immunity was mediated by CD8$^+$ T cells since immunized mice depleted of CD8$^+$ cells failed to resist tumor growth. *The Journal of Immunology,* **1999, 162: 6934–6941.**

Cancer cells form weakly immunogenic, tumor-associated Ags (TAAs)[4] (1–4) that can be recognized by CTLs. The TAAs are the products of dysregulated or mutant genes in the neoplastic cells that differ from the homologous genes in nonneoplastic cells of the same individual. Like other neoplasms, breast cancer cells form TAAs. The products of genes specifying HER-2/*neu* (5) MAGE-1 (6), BAGE (7), and MUC-1 (8–10) expressed by breast cancer cells have been identified as targets of CTLs. These may be only several representations of an undefined, and possibly large number of tumor Ags expressed by the malignant cells. Genetic instability is a characteristic phenotype of breast cancer and other types of malignant cells (11–15).

Under appropriate circumstances, tumor-specific cellular immune responses can be induced against TAAs expressed by neoplastic cells. The immune responses can be of sufficient magnitude to prolong the lives of tumor-bearing animals (16–20) and patients (21, 22). Genetic modification of tumor cells to secrete cytokines has been used as one means of augmenting the immunogenic properties of the malignant cells. Expression-competent genes for IL-2 (23–27), IL-4 (28), IL-6 (29), IL-7 (30), IL-12 (31), TNF-α (32, 33), IFN-α and IFN-γ (34, 35), and GM-CSF (36, 37), among others (38), have been introduced into neoplastic cells for this purpose. Immunizations with the cytokine-secreting, tumor cells resulted in cellular immune responses that were directed toward the malignant, but not the nonmalignant cells of the tumor-bearing host. Analogous tumor-specific responses were induced if the neoplastic cells used for the immunizations were modified to express syngeneic or allogeneic MHC determinants (39–42), or to express costimulatory molecules such as B7, required for activation of immune effector cells (43). However, the direct modification of cells from a primary neoplasm requires the establishment of a tumor cell line. This can be technically challenging, and may not always succeed. This is especially the case for breast cancer. Breast cancer cell lines are notoriously difficult to establish from primary breast neoplasms.

In other instances, defined tumor Ags or unfractionated tumor peptides have been used for tumor immunotherapy. However, few defined tumor Ags have been identified and cloned, and immunization with unfractionated tumor peptides requires large amounts of tumor if multiple immunizations are to be performed. Sufficient quantities of tumor tissue may not be available if patients are in clinical remission.

In this study, we tested an alternative approach. Classic studies indicated that transfection of DNA from one cell type can stably alter both the genotype and the phenotype of cells that take up the exogenous DNA. Wigler et al. (44), for example, reported stable integration of the gene for adenine phosphoribosyltransferase into mouse cells deficient in the enzyme by transfection of high m.w. genomic DNA from adenine phosphoribosyltransferase-positive mouse cells. A similar approach was used to convert thymidine kinase-deficient mouse cells to cells that expressed thymidine kinase by transfer of genomic DNA from a variety of thymidine kinase-positive tissues and cultured cells (45). In an analogous manner, Mendersohn et al. (46) reported that polio virus receptor-negative cells could be converted to cells that expressed the

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[2] E.d.Z. and V.C.-B. contributed equally to the investigation.

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[4] Abbreviation used in this paper: TAA, tumor-associated Ag.

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receptor by transfection of genomic DNA from receptor-positive cells. The products of single genes specifying the enzymes or membrane-associated determinants were expressed by subpopulations of the transfected cells.

We tested the hypothesis that a cellular vaccine capable of prolonging the survival of mice with breast cancer could be prepared by transfection of a highly immunogenic cell line with DNA from breast cancer cells. We reasoned that genes specifying numerous, undefined, weakly immunogenic TAAs would be expressed in a highly immunogenic form by the transfected cells, and that immunizations with the transfected cells would result in an immune response directed toward the breast cancer cells. We used two types of breast tumors, with analogous results. DNA from an adenocarcinoma of the breast that formed spontaneously in a C3H/He mouse was used to transfect a mouse fibroblast cell line that had been modified to secrete IL-2 and to express allogeneic class I MHC determinants (H-2K^b). A plasmid (pHyg) specifying resistance to hygromycin was included to allow selection of cells that had taken up the exogenous DNA. The antibiotic-resistant, transfected cells were then used to treat mice with breast cancer. The results indicated that mice immunized with the transfected fibroblasts developed generalized, cell-mediated immunity toward the breast cancer cells. The treated animals survived significantly longer than mice in various control groups, including mice with breast cancer treated by immunization with non-DNA-transfected fibroblasts. Similar results were obtained for mice bearing a mammary adenocarcinoma cell line (EO771) of C57BL/6J mouse origin treated with fibroblasts transfected with DNA from EO771 cells. The immunity was mediated by CD8$^+$ T lymphocytes since mice depleted of CD8$^+$ cells failed to resist tumor growth.

The augmented resistance to breast cancer in mice treated with fibroblasts transfected with breast cancer DNA points toward an analogous form of therapy for breast cancer patients.

Materials and Methods

Cell lines and experimental animals

Eight- to ten-week-old pathogen-free C3H/HeJ mice (H-2^k) and eight- to ten-week-old pathogen-free C57BL/6J mice (H-2^b) were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were maintained in the animal care facilities of the University of Illinois, according to National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. They were 8–12 wk old when used in the experiments. EO771 cells, a mammary adenocarcinoma cell line derived from a C57BL/6J mouse, were from the Tumor Repository of the Division of Cancer Treatment, Diagnosis and Centers of the National Cancer Institute (Frederick, MD). SB-1 cells were a breast adenocarcinoma that formed spontaneously in a C3H/HeJ mouse. B16 cells, a melanoma cell line originating in a C57BL/6J mouse, were from I. Fidler (MD Anderson, Houston, TX). EO771 cells were maintained by serial passage in histocompatible C57BL/6J mice. B16 cells were maintained by serial passage in C57BL/6J mice or at 37°C in a humidified 7% CO_2/air atmosphere in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Sigma, St. Louis, MO) and antibiotics (Life Technologies) (growth medium). LM cells, a fibroblast cell line of C3H/He mouse origin, were from the American Type Culture Collection (Manassas, VA). The cells were maintained at 37°C in a humidified 7% CO_2/air atmosphere in growth medium.

Modification of LM mouse fibroblasts for IL-2 secretion

LM fibroblasts were modified for IL-2 secretion by transduction with the retroviral vector pZipNeoSVIL-2 (from M. K. L. Collins, University College, London, U.K.) (LM-IL-2 cells). The vector, packaged in GP+*env* AM12 cells (from A. Bank, Columbia University, New York, NY), included a human IL-2 cDNA and a *neo*^{r} gene, both under control of the Moloney leukemia virus long terminal repeat. The *neo*^{r} gene conferred resistance to the aminoglycoside antibiotic, G418. Virus-containing supernatants of GP+*env* AM12 cells transfected with pZipNeoSVIL-2 were added to LM fibroblasts, followed by overnight incubation at 37°C in growth medium to which polybrene (Sigma; 5 μg/ml, final concentration) had been added. The cells were maintained for 14 days in growth medium containing 400 μg/ml G418 (Life Technologies). One hundred percent of nontransduced LM cells died in the medium supplemented with G418 during this period. Colonies of cells proliferating in the G418-containing growth medium were pooled for later use in the experiments. Every third, fourth, and fifth passage, the transduced cells were cultured in growth medium containing 400 μg/ml G418. IL-2 secretion by LM-IL-2 cells was detected by the capacity of supernatants from the transduced cells to sustain the growth of CTLL-2 cells, an IL-2-dependent cell line (47). Varying dilutions of the filtered culture supernatants (0.2 um nitrocellulose; Gelman, Ann Arbor, MI) were transferred to 96-well plates containing 1 × 10^4 CTLL-2 cells in a final volume of 200 μl of growth medium per well. After incubation for 16 h, 0.5 μCi [^{3}H]thymidine (Amersham, Arlington Heights, IL) was added to each well for additional 6 h of incubation. A standard curve was generated by adding varying amounts of human rIL-2 (Life Technologies) to an equivalent number of CTLL-2 cells. Afterward, the cells were collected onto glass fiber filters (Whittaker M.A. Products, Walkerville, MD) using a PhD multiple harvester (Microbiological Associates, Bethesda, MD). After washing with ethanol (95%), radioactivity in the insoluble fraction was measured in a liquid scintillation spectrometer (Packard Instrument, Downers Grove, IL). One unit of IL-2 resulted in half-maximal proliferation of CTLL-2 cells under these conditions.

Modification of LM-IL-2 cells for the expression of H-2K^b class I determinants

pBR327H-2K^b (Biogen Research, Cambridge, MA), a plasmid encoding MHC H-2K^b (48), was used to modify LM-IL-2 fibroblasts for the expression of H-2K^b determinants (LM-IL-2K^b cells). A total of 10 μg of pBR327H-2K^b and 1 μg of pBabePuro (from M. K. L. Collins), a plasmid conferring resistance to puromycin (49), was mixed with Lipofectin (Life Technologies), according to the supplier's instructions, and then added to 1 × 10^6 LM-IL-2 cells in 10 ml of DMEM without FBS. For use as a control, an equivalent number of LM-IL-2 cells was transfected with 1 μg of pBabePuro alone. The cells were incubated for 18 h at 37°C in a CO_2/air atmosphere, washed with DMEM, followed by the addition of 7 ml of growth medium. After incubation for 48 h, the cell cultures were divided and replated in growth medium supplemented with 3 μg/ml puromycin (Sigma), followed by incubation at 37°C for 7 additional days. The surviving colonies were pooled and tested by staining with specific FITC-conjugated Abs (described, below) for the expression of H-2K^b determinants. One hundred percent of nontransfected LM-IL-2 cells maintained in growth medium containing puromycin died during the 7-day period of incubation.

Immunofluorescent staining and cytofluorometric measurements

Quantitative immunofluorescent staining was used to detect the expression of H-2K^b determinants by LM-IL-2 cells transfected with pBR327H-2K^b. The measurements were performed in an Epic V flow cytofluorograph (Coulter Electronics, Hialeah, FL) equipped with a multiparameter data-acquisition and display system (MDADS). For the analysis, a single cell suspension was prepared from the monolayer cultures of puromycin-resistant cells with 0.1 mM EDTA in 0.1 M PBS, pH 7.4. The cells were washed with PBS containing 0.2% sodium azide and 0.5% FBS. Afterward, FITC-conjugated H-2K^b, H-2K^d, or H-2K^k mAbs (PharMingen, San Diego, CA), or FITC-conjugated IgG2a isotype serum (Dako, Carpenteria, CA) were added to the cells, followed by incubation at 4°C for 1 h. The cells were then washed with PBS containing 0.5% FBS and 0.2% sodium azide. One-parameter fluorescence histograms were generated by analyzing at least 1 × 10^4 cells. Background staining was determined by substituting cells stained with FITC-conjugated mouse IgG2a alone for cells stained with the specific Abs.

Depletion of mice of CD8$^+$ or CD4$^+$ T cells

mAbs were used to deplete naive C57BL/6J mice of CD8$^+$ or CD4$^+$ T cells. The mice were injected i.p. with the Ab-rich fraction obtained from ascites fluid containing anti-CD8 (83-23-5 mouse hybridoma) or from ascites fluid containing anti-CD4 (GK1.5 rat hybridoma) (both hybridomas were from Dr. K. Herald, University of Illinois at Chicago). The mice were injected i.p. with 0.3 ml (5 mg) of enriched 83-12-5 Abs, or i.p. with 0.2 ml (1 mg) of enriched GK1.5 Abs. Depletion of the relevant subset of T cells was verified by flow-cytofluorometric analysis of spleen cell suspensions taken 2 days after the injection of the enriched ascitic fluid. The depleted conditions were maintained in the remaining mice by injections of equivalent amounts of the appropriate Abs every 5 days until the experiments were concluded.

Transfection of LM-IL-2K^b cells with DNA from a breast carcinoma that arose spontaneously in a C3H/He mouse (SB-1), from EO771 breast carcinoma cells, or from B16 melanoma cells

Sheared, unfractionated DNA isolated (Qiagen, Chatsworth, CA) from a spontaneous mammary adenocarcinoma (SB-1) taken directly from a C3H/HeJ mouse, or from EO771 cells taken from a C57BL/6J mouse, or from B16 melanoma cells from in vitro culture, was used to transfect LM-IL-2K^b cells. The method described by Wigler et al. (45) was applied, as modified. Briefly, high m.w. DNA from each cell type was sheared by three passages through a 25-gauge needle. Afterward, 100 μg of the sheared DNA was mixed with 10 μg pHyg (from L. Lau, University of Illinois), a plasmid that encoded the *Escherichia coli* enzyme hygromycin B phosphotransferase (52), conferring resistance to hygromycin B. The sheared DNA and pHyg were then mixed with Lipofectin, according to the manufacturer's instructions (Life Technologies). The DNA/Lipofectin mixture was added to a population of 1×10^7 LM-IL-2K^b cells that had been divided into ten 100-mm plastic cell culture plates 24 h previously. Eighteen hours after addition of the DNA/Lipofectin mixture to the cells, the growth medium was replaced with fresh growth medium. For use as a control, DNA from the tumor cells was omitted, and 1 μg of pHyg alone, mixed with Lipofectin, was added to an equivalent number of LM-IL-2K^b cells. The same protocol was followed to transfect LM-IL-2 cells (not transduced with pBR327H-2K^b) with DNA from SB-1 cells. In each instance, the cells were maintained for 14 days in growth medium containing 600 μg/ml hygromycin B (Boehringer Mannheim, Indianapolis, IN). One hundred percent of LM-IL-2K^b or LM-IL-2 cells transfected with tumor-DNA alone maintained in the hygromycin growth medium died within this period. The surviving colonies (at least 2.5×10^4) of LM-IL-2K^b or of LM-IL-2 cells transfected with pHyg and DNA from the tumor cells, or with pHyg alone (LM-IL-2K^b cells), were pooled and used in the experiments.

Results

Modification of LM mouse fibroblasts for IL-2 secretion

A replication-defective retroviral vector, pZipNeoSVIL-2, was used to modify LM fibroblasts (H-2^k) for the secretion of IL-2. The vector specified the gene for human IL-2, along with a gene (neo^r) that conferred resistance to the neomycin analogue, G418. After selection in growth medium containing sufficient quantities of G418 to kill 100% of nontransduced cells, the surviving colonies were pooled and maintained as a cell line. Analysis of the culture supernatants indicated that 1×10^6 retrovirally transduced cells formed 150 U IL-2/10^6 cells/48 h, as determined by the capacity of the supernatants to sustain the growth of IL-2-dependent CTLL-2 cells. IL-2-secreting cells modified to express H-2K^b determinants (LM-IL-2K^b) and IL-2-secreting cells transfected with tumor DNA (described, below) formed equivalent quantities of IL-2. The culture supernatants of LM cells transduced with the IL-2-negative vector (pZipNeoSV(X), or of nontransduced LM cells failed to form detectable quantities of IL-2. Every third, fourth, and fifth passage, the IL-2-secreting cells were placed in medium containing 400 μg/ml G418. Under these conditions, similar quantities of IL-2 were detected in the culture supernatants of cells transduced with pZipNeoSVIL-2 for more than 6 mo of continuous culture (these data are not presented).

Modification of LM-IL-2 cells for the expression of MHC class I H-2K^b determinants

A plasmid, pBR327H-2K^b, was used to modify LM-IL-2 cells for the expression of H-2K^b determinants. LM-IL-2 cells were co-transfected with pBR327H-2K^b DNA along with pBabePuro DNA, used for selection. (A 10:1 ratio of pBR327H-2K^b DNA to pBabePuro DNA was used to increase the likelihood that cells that incorporated pBabePuro DNA took up pBR327H-2K^b DNA as well.) After selection in growth medium containing sufficient quantities of puromycin to kill the nontransduced cells, the surviving colonies were pooled and the cell number was expanded in vitro.

The expression of H-2K^b determinants by the modified cells was measured by quantitative immunofluorescent staining, using FITC-labeled mAbs for mouse H-2K^b determinants. As controls, aliquots of the puromycin-resistant cell suspension were incubated with FITC-labeled IgG2a isotype serum, or with FITC-labeled mAbs for H-2K^d determinants. As an additional control, the cells were incubated with FITC-labeled H-2K^b mAbs (LM cells are of C3H/He mouse origin). The mean fluorescent index of the puromycin-resistant LM-IL-2 cells stained with FITC-conjugated H-2K^b or FITC-conjugated H-2K^k mAbs (0.98 and 7.6, respectively) was significantly ($p < 0.001$) higher than that of cells stained with FITC-conjugated H-2K^d mAbs (Fig. 1). The MFI of cells stained with FITC-conjugated H-2K^d mAbs was approximately the same as that of cells stained with FITC-conjugated IgG2a isotype serum. The expression of H-2K^b determinants was a stable property of the transfected cells. The cells stained with equivalent intensity with FITC-conjugated H-2K^b mAbs after 3 mo of continuous culture (these data are not presented).

Tumor growth and survival of C57BL/6J mice injected with EO771 breast cancer cells and LM-IL-2K^b cells transfected with DNA from EO771 cells (LM-IL-2K^b/EO771)

C57BL/6J mice were highly susceptible to the growth of EO771 cells, a syngeneic breast cancer cell line. One hundred percent of mice injected with EO771 cells died from progressive tumor growth.

The effect of immunization with LM-IL-2K^b/EO771 cells on the growth of EO771 cells in C57BL/6J mice was determined by injecting naive mice into the fat pad of the breast with a mixture of EO771 cells and LM-IL-2K^b/EO771 cells, as described in the legend to Fig. 2. At the same time, the mice received an i.p. injection of 2×10^6 LM-IL-2K^b/EO771 cells alone. The mice then received two subsequent immunizations at weekly intervals with 2×10^6 LM-IL-2K^b/EO771 cells i.p. and an equivalent number of LM-IL-2K^b/EO771 cells injected into the same breast as first injected, without additional EO771 cells. As a control, naive C57BL/6J mice were injected into the breast with EO771 cells alone, followed by the subsequent injections of growth media. As additional controls, naive C57BL/6J mice were injected according to the same protocol with a mixture of EO771 cells and LM-IL-2K^b cells transfected with DNA from B16 melanoma cells (LM-IL-2K^b/B16), with EO771 cells and unmodified LM cells, or with EO771 cells and nontumor-DNA-transfected LM-IL-2K^b cells. The results

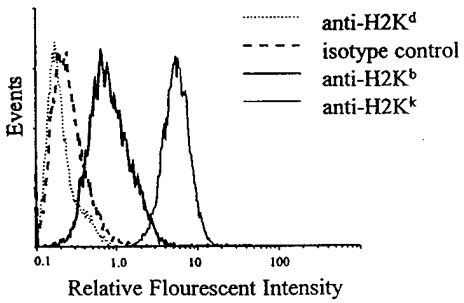

FIGURE 1. The expression of H-2K^b determinants by LM-IL-2 cells transduced with pBR327H-2K^b. A total of 1×10^4 LM-IL-2 cells transduced with the plasmid pBR327H-2K^b (LM-IL-2K^b cells) was incubated for 1 h at 4°C with FITC-conjugated anti-H-2K^b, anti-H-2K^k, or anti-H-2K^d mAbs, as described in *Materials and Methods*. The cells were then analyzed for fluorescent staining by flow cytofluorography.

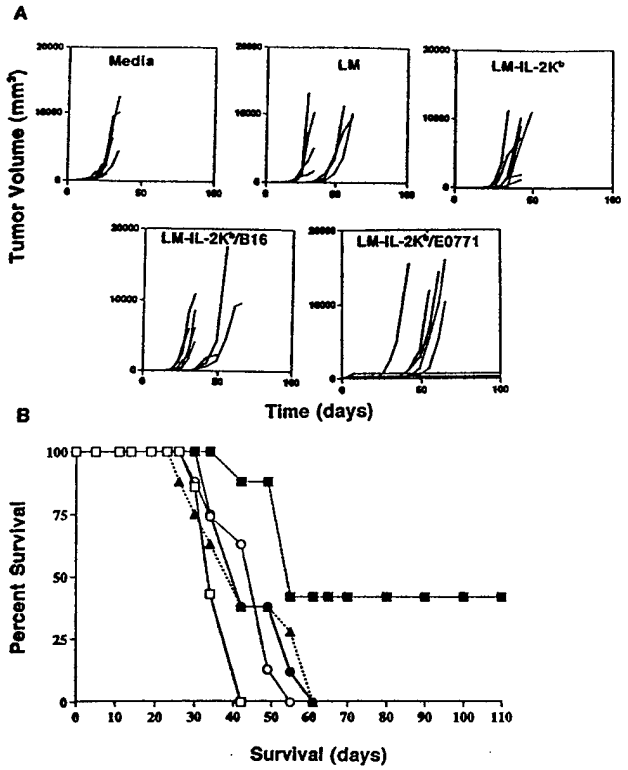

FIGURE 2. *A*, Tumor growth in C57BL/6J mice injected with EO771 breast cancer cells and LM-IL-2K^b/EO771 cells. C57BL/6J mice (seven per group) were injected into the fat pad of the breast with a mixture of 5×10^3 EO771 breast carcinoma cells and 2×10^6 LM-IL-2K^b/EO771 cells in a total volume of 200 μl. At the same time the mice also received an injection i.p. of 2×10^6 LM-IL-2K^b/EO771 cells in 200 μl alone, followed by two subsequent injections at weekly intervals of 2×10^6 LM-IL-2K^b/EO771 cells i.p. and 2×10^6 LM-IL-2K^b/EO771 cells into the fat pad of the same breast as first injected. As controls, other naive C57BL/6J mice were injected according to the same protocol with equivalent numbers of EO771 cells and unmodified LM cells, with EO771 cells and LM-IL-2K^b cells, with EO771 cells and LM-IL-2K^b/B16 cells, or with EO771 cells into the breast alone followed by subsequent injections of growth medium. Mean tumor volume was derived from two-dimensional measurements obtained with a dial caliper. The volume is equal to $0.4ab^2$, where a = length and b = width. *B*, Survival of C57BL/6J mice injected with EO771 breast carcinoma cells and LM-IL-2K^b/EO771 cells. C57BL/6J mice (seven per group) were injected into the fat pad of the breast with a mixture of 5×10^3 EO771 breast carcinoma cells and 2×10^6 LM-IL-2K^b/EO771 cells in a total volume of 200 μl. At the same time the mice also received an injection i.p. of 2×10^6 LM-IL-2K^b/EO771 cells in 200 μl alone, followed by two subsequent injections at weekly intervals of 2×10^6 LM-IL-2K^b/EO771 cells i.p. and 2×10^6 LM-IL-2K^b/EO771 cells into the fat pad of the same breast as first injected. As controls, other naive C57BL/6J mice were injected according to the same protocol with equivalent numbers of EO771 cells and LM cells, with EO771 cells and LM-IL-2K^b cells, with EO771 cells and LM-IL-2K^b/B16 cells, or with EO771 cells into the breast alone with subsequent injections of growth medium. Mean survival times: Mice injected with viable EO771 cells alone, 34.5 ± 5.8 days; mice injected with viable EO771 cells and LM cells, 41 ± 14 days; mice injected with viable EO771 cells and LM-IL-2K^b cells, 44 ± 9 days; mice injected with viable EO771 cells and LM-IL-2K^b/B16 cells, 46 ± 11 days; three mice injected with viable EO771 cells and LM-IL02K^b/EO771 cells, >110 days; mean survival times for remaining mice dying from progressive tumor growth, 54 ± 9. The p value for difference in survival of mice injected with viable EO771 cells and LM-IL-2K^b/EO771 cells, relative to survival of mice in each of the other groups, was <0.01. □, Injected with EO771 cells alone; ○, injected with EO771 cells and LM cells; ●, injected with EO771 cells and LM-IL-2K^b cells; ▲, injected with EO771 cells and LM-IL-2K^b/B16 cells; ■, injected with EO771 cells and LM-IL-2K^b/EO771 cells. The p values are as follows: $p < 0.01$ for difference in survival of mice injected with EO771 cells, and mice injected with EO771 cells and LM-IL-2K^b/EO771 cells; $p < 0.01$ for difference in survival of mice injected with EO771 cells and LM cells, and mice injected with EO771 cells and LM-IL-2K^b/EO771 cells; $p < 0.01$ for difference in survival of mice injected with EO771 cells and LM-IL-2K^b cells, and mice injected with EO771 cells and LM-IL-2K^b/EO771 cells; and $p < 0.01$ for difference in survival of mice injected with EO771 cells and LM-IL-2K^b/B16 cells, and mice injected with EO771 cells and LM-IL-2K^b/EO771 cells.

(Fig. 2*A*) indicate that the first appearance of tumor was significantly delayed ($p < 0.004$) in the group of mice injected with the mixture of EO771 cells and LM-IL-2K^b/EO771 cells, relative to that of mice in any of the other groups. Three mice in the group injected with EO771 cells and LM-IL-2K^b/EO771 cells failed to develop tumors and appeared to have rejected the breast cancer cells.

The development of resistance to EO771 cells in mice immunized with LM-IL-2K^b/EO771 cells was emphasized by the finding that the immunized mice survived significantly ($p < 0.01$) longer than mice in any of the various control groups, including mice injected with EO771 cells and LM-IL-2K^b cells transfected with DNA from B16 melanoma cells. Mice immunized with LM-IL-2K^b cells transfected with DNA from B16 cells failed to resist the growth of the breast cancer cells (Fig. 2*B*). In some instances, mice injected with EO771 cells and LM-IL-2K^b/EO771 cells survived indefinitely, more than 110 days. The injections of LM-IL-2K^b/EO771 cells were without apparent harm. Tumors failed to form in mice injected with LM-IL-2K^b/EO771 cells alone. Since LM cells express foreign histocompatibility determinants in C57BL/6J mice, it is likely that, like other foreign tissue grafts, the cells were rejected.

To determine whether the injections of LM-IL-2K^b/EO771 cells resulted in generalized, long-term immunity toward the breast cancer cells, surviving mice in the group immunized with EO771 cells and LM-IL-2K^b/EO771 cells received a second injection of EO771 cells 110 days after the first immunization. The presence of generalized, long-term immunity to the breast cancer cells was indicated by the finding that mice injected a second time with EO771 cells survived significantly ($p < 0.02$) longer than naive mice injected with an equivalent number of EO771 cells alone (Fig. 3).

CD8$^+$ cells mediate immunity to breast cancer in mice immunized with fibroblasts transfected with DNA from breast cancer cells

T cell depletion was used to determine the subset of T cells that mediated resistance to tumor growth in mice immunized with the DNA-transfected cells. In the experiment, T cell depletion was accomplished by injecting C57BL/6J mice i.p. with CD8$^+$ or CD4$^+$ mAbs, as described in *Materials and Methods*. Two days later, the mice received a second injection of the Abs, followed by an injection into the fat pad of the breast with a mixture of 5×10^3 EO771 cells and 2×10^6 LM-IL-2K^b/EO771 cells. The mice received two subsequent injections of equivalent numbers of LM-IL-2K^b/EO771 cells and additional injections of the mAbs, as described. As indicated, the first appearance of tumor and survival of immunized mice depleted of CD8$^+$ cells (Fig. 4*Aa*) was not significantly different from the first appearance of tumor and survival of mice injected with EO771 cells alone (Fig. 4*Ab*). Depletion of CD4$^+$ cells had no apparent effect on resistance to tumor growth. The first appearance of tumor and survival of CD4$^+$ T cell-depleted mice injected with EO771 cells and LM-IL-2K^b/EO771 cells (Fig. 4*Ac*) was not significantly different from the first appearance of tumor and survival of mice injected with EO771 cells

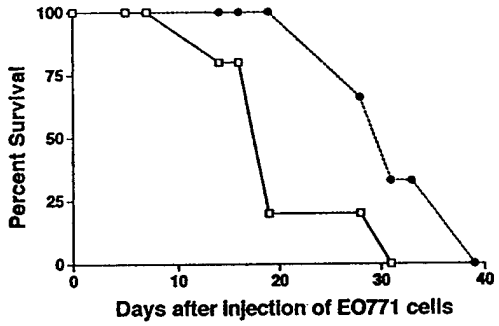

FIGURE 3. Survival of C57BL/6J mice surviving a prior injection of EO711 cells and LM-IL-2K^b/EO771 cells injected with EO771 cells alone. Three C57BL/6J mice surviving 110 days after the prior injection of EO711 cells and LM-IL-2K^b/EO771 cells were injected into the fat pad of the breast a second time with 5×10^3 EO771 cells alone. As a control, five naive C57BL/6J mice were injected into the fat pad of the breast with an equivalent number of EO771 cells; $p < 0.02$ for the difference in survival of mice in the two groups. ●, Surviving mice injected with EO711 cells; □, naive mice injected with EO711 cells.

and LM-IL-2K^b/EO771 cells alone (Fig. 4*Ad*). Thus, depletion of CD8$^+$ T cells, but not CD4$^+$ cells, affected the animals' capacity to resist the growth of the breast cancer cells in mice immunized with the DNA-transfected cells.

CD8$^+$ T cell depletion had analogous effects on the survival of mice injected with the breast cancer cells and the DNA-transfected fibroblasts. As indicated (Fig. 4*B*), the survival of CD8$^+$-depleted mice injected with EO771 cells and LM-IL-2K^b/EO771 cells was significantly ($p < 0.01$) less than the survival of nondepleted mice injected with EO771 cells and LM-IL-2K^b/EO771 cells alone. It was not significantly different from the survival of non-T cell-depleted mice injected with EO771 cells alone. In contrast, depletion of CD4$^+$ cells had no effect on survival. The survival of mice depleted of CD4$^+$ cells injected with EO771 cells and LM-IL-2K^b/EO771 cells was not significantly different from that of non-T cell-depleted mice injected with EO771 cells and LM-IL-2K^b/EO771 cells alone.

Thus, depletion of CD8$^+$ but not CD4$^+$ cells affected both tumor growth and survival of the immunized mice with breast cancer.

Survival of C3H/HeJ mice injected with cells from a spontaneous adenocarcinoma of the breast (SB-1) and LM-IL-2K^b cells transfected with DNA from SB-1 cells

Specific partial immunity toward EO771 cells, a breast cancer cell line, was generated in C57BL/6J mice immunized with semiallogeneic, IL-2-secreting mouse fibroblasts transfected with DNA from EO771 cells. The same protocol was followed to determine whether an analogous response would be obtained in mice immunized with the modified fibroblasts transfected with DNA taken directly from a spontaneous breast adenocarcinoma arising in a C3H/HeJ mouse.

C3H/HeJ mice develop breast cancer spontaneously. A tumor that developed in the breast of a 12-mo-old mouse was excised and used as a source of DNA to develop the vaccine. Histologic sections indicated that it was an adenocarcinoma. In addition, naive C3H/HeJ mice had no apparent resistance to the growth of the breast cancer cells. One hundred percent of mice injected with 1×10^4 SB-1 cells into the fat pad of the breast died from progressive tumor growth in approximately 30 days.

The effect of immunization with LM-IL-2K^b cells transfected with DNA from the spontaneous breast neoplasm (SB-1 cells) on

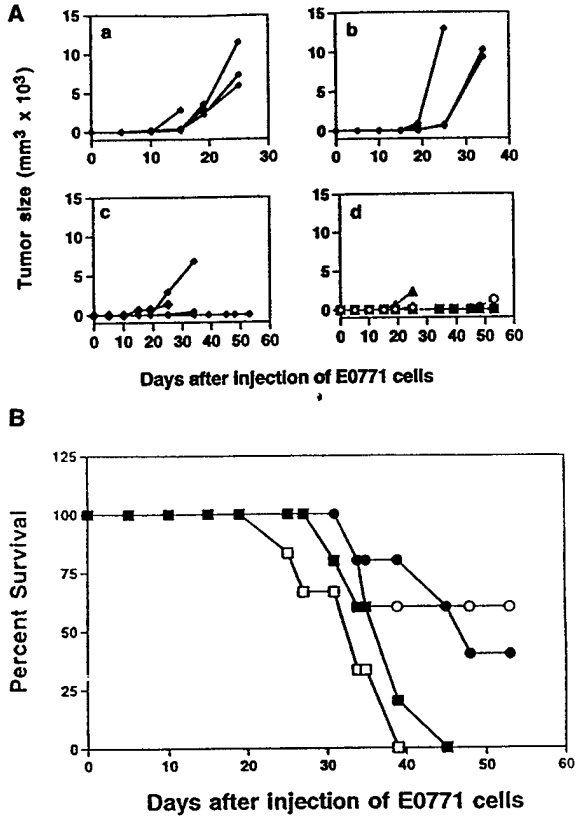

FIGURE 4. *A*, Tumor growth in C57BL/6J mice depleted of CD8$^+$ T lymphocytes injected with a mixture of EO711 breast cancer cells and LM-IL-2K^b/EO771 cells. C57BL/6J mice (seven per group) were injected i.p. with CD4$^+$ (group c) or CD8$^+$ (group b) mAbs, as described in *Materials and Methods*. Two days later, the mice were injected into the fat pad of the breast with a mixture of 5×10^3 EO711 breast carcinoma cells and 2×10^6 LM-IL-2K^b/EO711 cells in a total volume of 200 μl. At the same time, the mice also received an injection i.p. of 2×10^6 LM-IL-2K^b/EO711 cells in 200 μl alone. The mice received additional injections of the relevant Abs as described, and two subsequent injections at weekly intervals of 2×10^6 LM-IL-2K^b/EO771 cells i.p. and 2×10^6 LM-IL-2K^b/EO771 cells into the fat pad of the same breast as first injected. As controls, other C57BL/6J mice (group c) were injected according to the same protocol with EO771 cells and LM-IL-2K^b/EO771 cells, but did not receive mAbs, or with equivalent numbers of EO771 cells into the breast alone followed by subsequent injections of growth medium (group d). Mean tumor volume was derived from two-dimensional measurements obtained with a dial caliper. The volume is equal to $0.4ab^2$, where a = length, and b = width. *B*, Survival of C57BL/6J mice depleted of T cells injected with a mixture of EO771 breast carcinoma cells and LM-IL-2K^b/EO771 cells. The same protocol as described in *A* was followed except that survival of the Ab-treated mice was determined. ■, Injected with CD8$^+$ Abs, EO771 cells, and LM-IL-2K^b/EO771 cells; ●, injected with CD4$^+$ Abs, EO771 cells, and LM-IL-2K^b/EO771 cells; □, injected with EO771 cells alone; ○, injected with EO771 cells and LM-IL-2K^b/EO771 cells alone.

the growth of the breast cancer cells was determined by injecting naive C3H/HeJ mice into the fat pad of the breast with SB-1 cells and LM-IL-2K^b/SB-1 cells, and i.p. with LM-IL-2K^b/SB-1 cells alone. As previously, the mice received two subsequent injections i.p. and two subsequent injections into the same breast as first injected with the same number of LM-IL-2K^b/SB-1 cells. The results (Fig. 5*A*) indicated that the time to first appearance of a palpable tumor in the breasts of mice injected with the mixture of SB-1 cells and LM-IL-2K^b/SB-1 cells was significantly delayed ($p < 0.006$), relative to the first appearance of tumor in mice injected

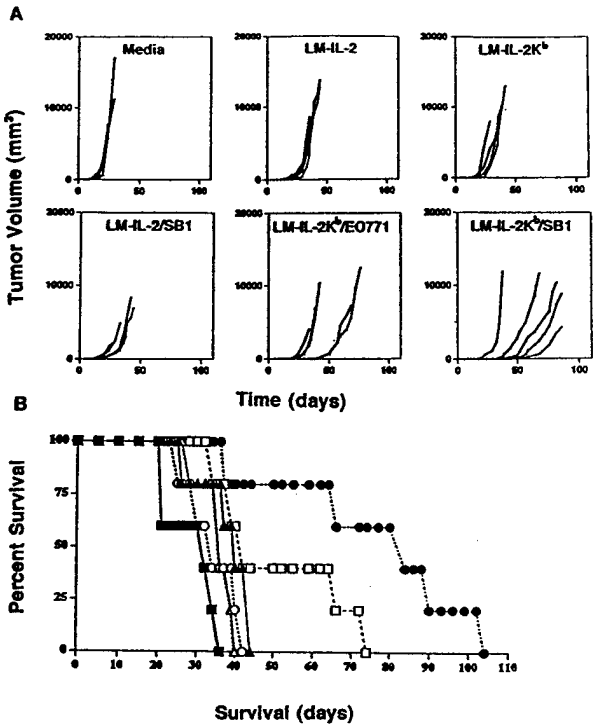

FIGURE 5. *A*, Tumor growth in C3H/HeJ mice injected with cancer cells from a spontaneous breast neoplasm (SB-1) and LM-IL-2K^b cells transfected with DNA from SB-1 cells. C3H/HeJ mice (five per group) were injected into the fat pad of the breast with a mixture of 1×10^6 SB-1 cells and 2×10^6 LM-IL-2K^b/SB-1 cells. At the same time, the mice received an injection i.p. of 2×10^6 LM-IL-2K^b/SB-1 cells alone, followed by two subsequent injections. As controls, the mice were injected according to the same protocol with equivalent numbers of SB-1 cells alone, with SB-1 cells and LM-IL-2 cells, with SB-1 cells and LM-IL-2K^b cells, with SB-1 cells and LM-IL-2/SB-1 cells, or with SB-1 cells and LM-IL-2K^b/EO771 cells. The mice were injected i.p. twice more, at weekly intervals, with the same number of modified cells as in the initial injections, but without additional SB-1 cells. Mean tumor volume was derived from two-dimensional measurements obtained with a dial caliper. *B*, Survival of C3H/HeJ mice injected with a mixture of SB-1 breast carcinoma cells and LM-IL-2K^b/SB-1 cells. C3H/HeJ mice (five per group) were injected into the fat pad of the breast with a mixture of 5×10^3 SB-1 cells and 2×10^6 LM-IL-2K^b/SB-1 cells in a total volume of 200 μl. A the same time the mice received an injection i.p. of 2×10^6 LM-IL-2K^b/SB-1 cells in 200 μl alone, followed by two subsequent injections at weekly intervals of 2×10^6 LM-IL-2K^b/SB-1 cells i.p. and 2×10^6 LM-IL-2K^b/SB-1 cells into the fat pad of the same breast as first injected. As controls, other naive C57BL/6J mice were injected according to the same protocol with equivalent numbers of SB-1 cells and LM-IL-2 cells, with SB-1 cells and LM-IL-2K^b cells, with SB-1 cells and LM-IL-2/SB-1 cells, with SB-1 cells and LM-IL-2K^b/EO771 cells, or with SB-1 cells into the breast alone, without subsequent injections. Mean survival times: Mice injected with SB-1 cells alone, 29 ± 7 days; with SB-1 cells and LM-IL-2 cells, 38 ± 8 days; with SB-1 cells and LM-IL-2K^b cells, 34 ± 7 days; with SB-1 cells and LM-IL-2/SB-1 cells, 36 ± 5 dys; with SB-1 cells and LM-IL-2K^b/EO771 cells, 51 ± 18 days; with SB-1 cells and LM-IL-2K^b/SB-1 cells, 76 ± 26 days. Survival of mice injected with SB-1 cells and LM-IL-2K^b/SB-1 cells, relative to survival of mice in each of the other groups, $p < 0.02$ for difference in survival of mice injected with SB-1 cells alone and mice injected with SB-1 cells and LM-IL-2K^b/EO771 cells. ■, Injected with SB-1 cells alone; △, injected with SB-1 cells and LM-IL-2 cells; ○, injected with SB-1 cells and LM-IL-2K^b cells; ▲, injected with SB-1 cells and LM-IL-2/SB-1 cells; □, injected with SB-1 cells and LM-IL-2K^b/EO771 cells; ●, injected with SB-1 cells and LM-IL-2K^b/SB-1 cells.

with SB-1 cells alone. Once the breast neoplasms first appeared, the rate of tumor growth (two-dimensional measurements) in the treated and untreated groups was approximately the same.

Consistent with the delayed appearance of tumor in the treated group, mice injected with SB-1 cells and LM-IL-2K^b/SB-1 cells survived significantly ($p < 0.006$) longer than mice injected with SB-1 cells alone (Fig. 5*B*). No tumors formed at immunization sites injected with LM-IL-2K^b/SB-1 cells alone.

As controls, naive C3H/HeJ mice were injected according to the same protocol with SB-1 cells and nontransfected LM-IL-2 cells, with SB-1 cells and nontransfected LM-IL-2K^b cells, or with SB-1 cells and syngeneic LM-IL-2 cells transfected with DNA from SB-1 cells (LM-IL-2/SB-1). As indicated (Fig. 5*A*), with the exception of two mice in the group injected with SB-1 cells and LM-IL-2K^b/EO771 cells, the first appearance of tumor, rate of tumor growth, and survival of mice in each group were approximately the same as that of mice injected with SB-1 cells alone. Thus, the greatest immunotherapeutic benefit was in the group of mice injected with the mixture of SB-1 cells and semiallogeneic LM-IL-2K^b cells transfected with genomic DNA from SB-1 cells.

As a means of determining whether immunizations with LM-IL-2K^b cells transfected with DNA from EO771 cells conferred immunity to SB-1 cells, naive C3H/HeJ mice were injected with a mixture of SB-1 cells and LM-IL-2K^b/EO771 cells. As indicated (Fig. 5*B*), although mice injected with SB-1 cells and LM-IL-2K^b/EO771 cells survived longer than mice injected with SB-1 cells alone, they died in significantly ($p < 0.01$) shorter intervals than mice injected with SB-1 cells and LM-IL-2K^b cells transfected with DNA from the same breast cancer.

Discussion

The extraordinarily high incidence of breast cancer in women, approximately one in eight will develop the disease at some point in her life, created an urgent need for new and innovative forms of therapy. Immunotherapeutic approaches, designed to stimulate immunity to autologous tumor, are under active investigation for a number of different types of cancers. The theoretical basis underlying this form of treatment is that neoplastic cells form unique TAAs that can be recognized by CTL, and that cellular immunity to TAAs can follow immunization with tumor vaccines. Malignant cells in the patient can become targets of immune-mediated attack. Like other neoplasms, breast cancer cells form TAAs, several of which have been identified (5–8). However, Ags associated with the proliferating malignant cells are insufficiently immunogenic to generate an effective immune response. Proliferating breast cancer cells fail to elicit antitumor immune responses that can control tumor cell growth.

In this study, we transferred high m.w. DNA from breast cancer cells into a mouse fibroblast cell line to develop a breast cancer vaccine that was effective in the treatment of breast cancer in mice. This approach was based on prior studies that indicated that the introduction of high m.w. genomic DNA from one cell type altered both the genotype and the phenotypic characteristics of the cells that took up the exogenous DNA. This was the case for transfer of single genes specifying enzymes or membrane receptors (44–46). The gene products were expressed by subpopulations of the transfected cells. In an analogous manner, transfer of breast cancer DNA into a highly immunogenic cell line resulted in a cellular vaccine that was effective in the treatment of breast cancer in mice. The results were consistent with the expression in a highly immunogenic form of undefined breast cancer-associated Ags by a subpopulation of the DNA-transfected cells.

Mouse fibroblasts were chosen as the platform for expression of the breast cancer-associated Ags, for several important reasons. The cells, maintained as a cell line in vitro, were readily transfected, using conventional laboratory procedures. And, since the exogenous DNA was replicated as the cells divided, the number of transfected cells could be expanded as might be required for multiple immunizations of the tumor-bearing mice. In addition, like dendritic cells, fibroblasts can act as efficient APCs (53, 54). They constitutively express B7.1, a costimulatory molecule required for T cell activation (55). Class I cellular antitumor immune responses were generated in tumor-bearing mice immunized with fibroblasts transfected with tumor DNA (56, 57).

In this study, DNA was isolated from an adenocarcinoma of the breast that arose spontaneously in a C3H/HeJ mouse (H-2^k). DNA from the breast cancer cells was used to transfect LM cells, a mouse fibroblast cell line of C3H/He mouse origin. To increase their nonspecific immunogenic properties, and to ensure rejection, the fibroblasts were modified to express foreign (allogeneic) H-$2K^b$ determinants, and to secrete IL-2 before they were transfected with the tumor DNA. Antitumor immune responses were generated in mice immunized with the transfected cells. The first appearance of tumor was delayed and the mice survived significantly longer than mice in various control groups, including mice injected with the breast cancer cells and transfected fibroblasts that formed syngeneic MHC determinants alone.

An analogous study was conducted using IL-2-secreting LM fibroblasts modified to express H-$2K^b$ determinants that were transfected with DNA from EO771 cells, a breast cancer cell line of C57BL/6 mouse origin. H-$2K^b$ determinants were syngeneic class I MHC determinants in C57BL/6J mice, providing a restriction element for direct Ag presentation to CTLs of the host (53). Like the survival of C3H/HeJ mice with breast cancer treated by immunization with fibroblasts transfected with breast cancer DNA, C57BL/6J mice injected with EO771 cells and LM-IL-$2K^b$ cells transfected with DNA from EO771 cells survived significantly longer than mice in various control groups, including mice injected with EO771 cells and modified fibroblasts transfected with DNA from mouse melanomas, an unrelated tumor. Some of the mice immunized with the breast cancer DNA-transfected fibroblasts appeared to have rejected the breast cancer cells and survived indefinitely. Immunity failed to develop in mice depleted of $CD8^+$ cells, indicating the essential role of this subset of T cells in mediating tumor rejection.

Whether or not the immunity in mice injected with the DNA-transfected cells was local, or systemic, was not determined. The injections were administered in the vicinity of the tumor. However, several lines of evidence lead us to speculate that systemic immunity to the breast cancer cells may have been engendered by the immunizations. In addition to the involvement of $CD8^+$ cells in mediating the antitumor response, the survival of mice treated previously by immunization with the DNA-based vaccine, and then rechallenged 4 mo later by a second injection of the breast cancer cells was significantly prolonged. Finally, the failure of non-DNA-transfected cells or of cells transfected with DNA from a heterologous tumor (B16 melanoma) to induce an antibreast cancer immune response is consistent with a systemic response. Further studies are required to establish this point.

We conclude that an array of undefined breast cancer-associated Ags was expressed by the modified fibroblasts transfected with breast cancer DNA. No attempt was made to identify TAAs expressed by the transfected cells. The identification of tumor Ags is technically challenging and may not be required in the treatment of breast cancer patients. Immunization with a vaccine that expresses multiple TAAs may have advantages over immunization with one, or even several defined Ags. Immunotherapy with defined Ags may not eliminate the entire malignant cell population, as some tumor cells may fail to express the Ag(s) chosen for immunization.

Transfection of tumor DNA into a highly immunogenic cell line has other important advantages. The amount of tumor DNA required to prepare the vaccine can be small, since the transferred DNA is replicated as the cells divide. In addition, a tumor cell line does not have to be established if the patient's own tumor is to be genetically modified for immunization. Tumor DNA can be readily obtained from primary neoplasms. Furthermore, the cells used as recipients of the tumor DNA can be modified in advance for special properties, such as identity with the patient for shared class I determinants, or to secrete one or more cytokines, to further augment their immunogenic properties.

Surprisingly, the number of transfected cells that expressed the products of genes specifying TAAs was sufficient to induce the antitumor immune response. Our observation that antitumor immune responses followed immunizations with the transfected cells may be an indication that multiple, and possibly large numbers of immunologically distinct TAAs, the products of multiple altered genes, were present within the population of breast cancer cells. The prolonged survival of mice injected with cells from a spontaneous breast neoplasm (SB-1) treated with a vaccine prepared with DNA from an independently arising breast cancer cell line (EO771) suggests that the two breast cancers share Ags in common.

The results reported in this work raise the possibility that a human fibroblast cell line that shares identity at one or more MHC class I alleles with the cancer patient may be readily modified to provide immunologic specificity for TAAs expressed by the patient's neoplasm. The data suggest that an optimum response can be obtained if the cellular immunogen is prepared using DNA from the patient's own tumor. Transfection of the cell line with DNA from the neoplastic cells may provide a practical alternative to the modification of autologous malignant cells for the purposes of generating an immunogen that is useful in the overall management of the patient's disease.

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Expression of B7.1 in a MUC1-expressing mouse mammary epithelial tumour cell line inhibits tumorigenicity but does not induce autoimmunity in *MUC1* transgenic mice

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SUMMARY

The MUC1 epithelial mucin, which is overexpressed and aberrantly glycosylated in breast and other carcinomas, is also expressed on the apical surface of most normal glandular epithelial cells. Since clinical trials evaluating the efficacy of *MUC1*-based vaccines have been initiated in breast cancer patients, it is important to address the question of whether an effective immune response to the cancer associated mucin can be generated without inducing autoimmunity. Since non-classic cytotoxic T lymphocyte (CTL) responses to MUC1 have been reported, it is also relevant to examine the role of costimulatory molecules in the effective presentation of MUC1 based antigens. We have therefore looked at the effect of expressing B7.1 on the tumorigenicity of a MUC1 expressing mammary epithelial cell line (410.4) in a transgenic mouse expressing MUC1 on its normal glandular epithelial tissues. Coexpression of B7.1 with MUC1 in 410.4 cells resulted in a dramatic inhibition of tumour growth which depended on the activity of $CD4^+$ and $CD8^+$ T cells. The epithelial tissues in the transgenic mice able to reject the B7.1, MUC1-expressing tumours showed no evidence of degeneration and the mice survived their normal life span. The results demonstrate that an immune response to the MUC1 antigen can be induced in *MUC1* transgenic mice and that presentation of the antigen, whether directly or by cross-priming, is markedly enhanced by coexpression of B7.1.

INTRODUCTION

The human *MUC1* gene codes for a type I membrane glycoprotein that is normally expressed on the apical surface of most glandular epithelial cells, but which is upregulated and underglycosylated in breast and other carcinomas.[1] The extracellular domain of the MUC1 mucin is made up largely of exact tandem repeats of 20 amino acids, each repeat containing sites for *O*-linked glycosylation.[2] The shorter carbohydrate side chains which are added in breast cancers result in the exposure of normally cryptic peptide epitopes (such as that recognized by the antibody SM3), and the creation of tumour-associated carbohydrate epitopes.[3] Humoral responses to MUC1 have been detected in breast and other cancer patients[4,5] while both major histocompatibility complex (MHC)-unrestricted and MHC-restricted cytotoxic T lymphocyte (CTL) responses have also been reported.[6–8] Several clinical studies using MUC1-based antigens have been initiated.[9] However, the importance of the different components of the immune response remains unclear.

Because the MUC1 antigen is expressed by normal glandular epithelial cells, the question of whether autoimmunity would occur if the immune responses to the mucin were effective is a question which needs to be addressed. A dominant immunogenic domain of MUC1 is in the tandem repeat sequence,[2,10,11] which has a different sequence in the mouse homologue, Muc1,[12] making the use of the usual mouse strains for the preclinical studies inappropriate. To address this and related questions, we have developed a transgenic mouse expressing the human MUC1 mucin on glandular epithelial tissues with the same profile of expression as is seen in humans.[13] Moreover, the differences in glycosylation of MUC1 seen between normal and malignant breast are also evident in the transgenic mouse: The SM3 epitope is minimally expressed on the mucin expressed by normal glandular tissues,[13] but is expressed on both transplantable tumours expressing MUC1[14] and on spontaneous tumours developing in transgenics cross-fostered on an MMTV carrying mouse strain.[15] Thus, the MUC1 transgenic mouse provides a suitable model for preclinical testing of MUC1-based vaccines.

Introduction of a gene coding for costimulatory molecules such as B7.1 can enhance the immunogenicity of mouse tumour cell lines and thus reduce their tumorigenicity.[16,17] To

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ascertain whether an effective immune response to the MUC1 mucin can be obtained in the *MUC1* transgenic mouse, we have examined the effect of coexpressing the B7.1 costimulatory molecule with MUC1 on the tumorigenicity of a mouse mammary epithelial cell line in these mice. Our results indicate that while coexpression of the B7.1 molecule markedly decreases the tumorigenicity of the MUC1-expressing 410.4 mammary epithelial tumour cell, expression of MUC1 or B7.1 alone had no such effect. The effects of B7.1 expression are related to T-cell responses as no difference in tumorigenicity was seen in B7.1/MUC1-expressing cells in the nude mouse. Moreover, *in vivo* depletion of $CD4^+$ and $CD8^+$ T cells in the transgenic mice abolished the protective effect of coexpression of MUC1 and B7.1. Significantly, no evidence of tissue degeneration was seen in the epithelial tissues expressing MUC1 and the mice lived out their natural life span with no evidence of disease.

MATERIALS AND METHODS

Development of 410.4-derived cell lines

The 410.4H and E3 cell lines were developed from the 410.4 cells[18] by transfection of the hygromycin resistance gene with (E3) or without (410.4H) the MUC1 gene.[14] Calcium phosphate transfection of 410.4H and E3 cells was perfomed using the pbabe-neo gene (kind gift of H. Land, ICRF, London) with or without the cDNA encoding murine B7.1 contained in the plasmid πLN (a kind gift from P. Linsley, Seattle, WA). The nomenclature of the selected clones (which were grown in Dulbecco's E4 medium containing 600 μg/ml G418, 200 μg/ml hygromycin and 10% fetal calf serum (FCS)) is illustrated in Fig. 1.

Growth of cell lines in transgenic or nude mice

The transgenic mice homozygous for the *MUC1* gene (designated *Sac*II) are $H2^k$.[13] These mice were crossed with BALB/c mice to produce F1 hybrids (haplotype H-2^{kd}) to accept the 410.4 tumour cell derivatives (haplotype H-2^d). *Sac*II transgenic × BALB/c F1 mice, or nude mice aged between 6 and 14 weeks old were injected subcutaneously with a suspension of cells (in phosphate buffered saline (PBS)), from one of the 410.4-derived clones. Preliminary titrations in *Sac*II BALB/c mice showed that 10^5 was the lowest number of cells which consistently induced tumours (the parental tumour, the clone 410.4-H.neo or the MUC1-expressing clone). Mice were examined three times per week and tumour size estimated by measuring the largest diameter and its perpendicular. Mice were euthanized when the largest diameter reached 15 mm, the tumour ulcerated, or the mouse appeared sick. All experiments were conducted according to Home Office Guidelines under an approved project licence (JTP).

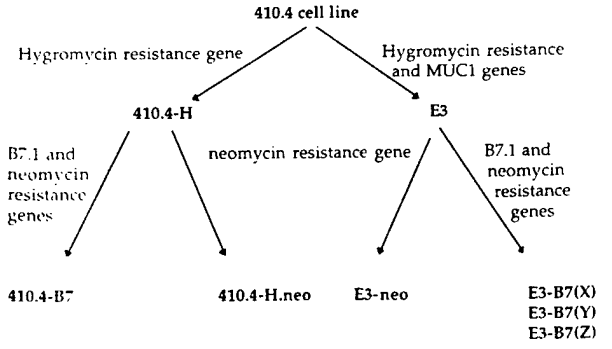

Figure 1. Development and nomenclature of 410.4-derived cell lines transfected with MUC1, B7.1 and selectable markers. For details of transfection and clone isolation see methods.

In vivo *depletion of $CD4^+$ and $CD8^+$ cells*

Five- to 6-week-old *Sac*II × BALB/c F1 mice were thymectomized under general anaesthetic and randomized into one of four groups. One week later they underwent T-cell subset depletion by intravenously injecting the relevant anti-T-cell antibodies in a volume of 200 μl of PBS, three times over a five-day period.[19] $CD4^+$ depletion was achieved by using 1 mg of the synergistic pair of antibodies YTS 191.1.2 (a rat immunoglobulin G2b (IgG2b) antimouse CD4 antibody, epitope a) and YTA 3.1.2 (a rat IgG2b antimouse CD4 antibody, epitope b). $CD8^+$ depletion was achieved by using 1 mg of the synergistic pair of antibodies YTS 169.4.2·1 (a rat IgG2b antimouse CD8α chain, antibody) and YTS 156.7.7 (a rat IgG2b antimouse CD8β chain (or α/β complex) antibody). $CD4^+/CD8^+$ depletion was achieved by using a combination of all the four antibodies to a total of 2 mg of antibody. Mice in the control group received 1 mg of the irrelevant antibody PYLT1 (against the polyoma virus large T antigen). All mice tested had successfully been depleted of the relevant subsets as tested in the spleens of mice killed before injection of the tumour cell lines. One week following T-cell depletion each mouse received a subcutaneous injection of 1×10^5 E3-B7(Z) cells.

Immunoperoxidase staining of sections

Tumours were fixed in methacarn (60% methanol: 30% chloroform: 10% acetic acid) for 4 hr, washed in 70% ethanol, and processed for wax-embedding. Sections were stained with biotinylated affinity purified monoclonal antibodies SM3 (50 μg/ml) or HMFG-1 (100 μg/ml) as previously described.[15]

Examination of tissues for evidence of Autoimmunity

Mice given subcutaneous injections into the flank of 1×10^5 cells of E3-B7 cells were euthanized on day 12 and at monthly intervals following the initial injection. The tumours and organs were analysed histologically for lymphocyte infiltration and evidence of tissue destruction.

Flow cytometry

Cells were screened for membrane expression of murine B7.1 by staining with CTLA4-immunoglobulin.[20] 5×10^5 cells suspended in 100 μl of neat supernatant from the CTLA4-immunoglobulin hybridoma (gift from Dr P. Lane, Basel, Switzerland) were incubated for 1 hr on ice, washed 3 times in cold PBS and resuspended in 100 μl of fluoroscein isothiocyanate (FITC)-conjugated rabbit antihuman antibody (Dako, Denmark, diluted 1:40) for 1 hr. After washing, cells were resuspended in 500 μl of PBS and 5000 cells analysed by a Becton-Dickinson fluorescence-activated cell sorter (FACscan flow cytometer; Becton-Dickinson, Oxford, UK). The clones were also screened in a similar manner for expression of MUC1 with the HMFG-1 mouse monoclonal antibody directed to an epitope in the tandem repeat of MUC1.

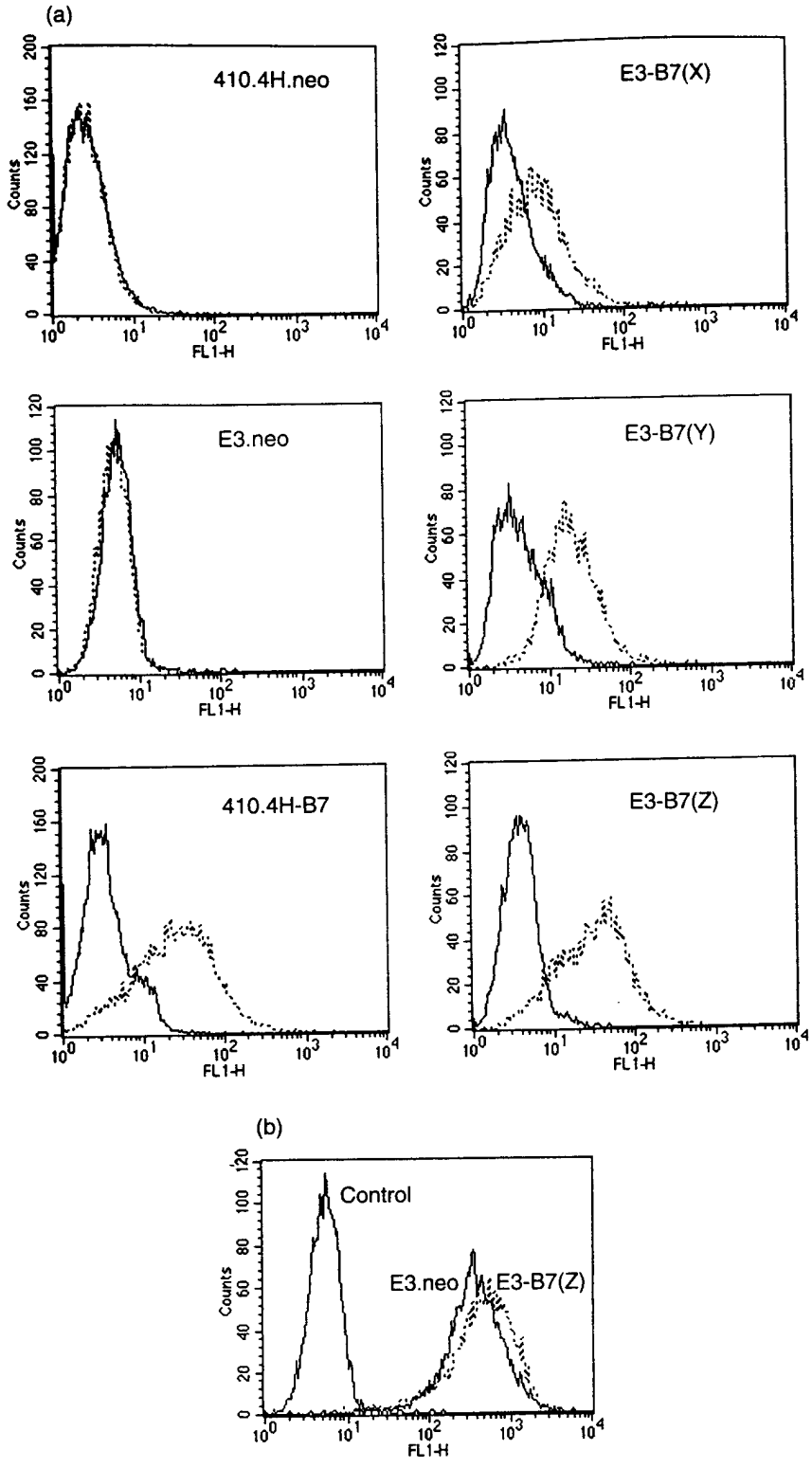

Figure 2. (a) Surface expression of B7.1 on the B7.1 transfected lines 410.4-B7, E3-B7(X), E3-B7(Y) and E3-B7(Z) as determined by FACS analysis. CTLA-4 binding (...); control (—). (b) Surface expression of the MUC1 antigen on E3 neo and E3-B7(Z) as determined by FACS analysis.

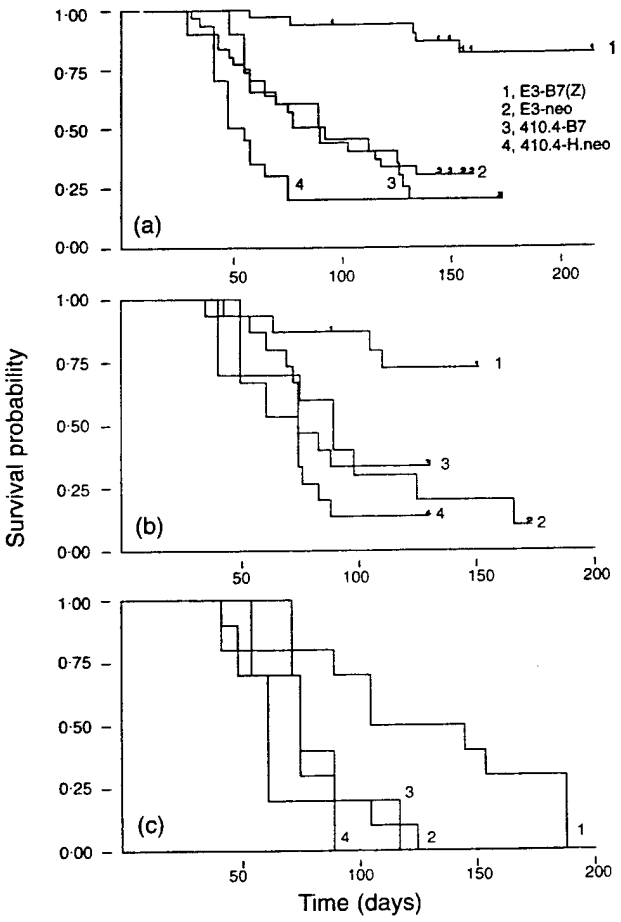

Figure 3. Kaplan–Meier survival curves of mice injected with various doses of 410.4 derived lines. F1 hybrid mice from the MUC1 transgenic mice crossed with BALB/c mice were injected with 10^5 (a), 2×10^5 (b) or 10^6 (c) cells and the development of tumours followed as described in Materials and Methods. The curves represent composites of several experiments, and *P*-values for the log rank heterogeneity test were <0·0001 (a), <0·02 (b) and <0·03 (c).

Cytotoxic T-cell assays

*Sac*II transgenic × BALB/c F1 mice were immunized, in the flank, with 1×10^5 cells of the B7/MUC1 expressing tumour E3-B7(Z). Four to five weeks later splenocytes were isolated and stimulated *in vitro* with mitomycin-C-treated MUC1-expressing P815 cells and after 5 days, the cells were tested for their ability to lyse P815-MUC1 cells in a standard chromium release assay[21] using effector:target ratios from 50:1 to 1·5:1. Control samples to which no lymphocytes had been added, indicated the level of spontaneous lysis and samples to which 100 µl of 10% Triton-X-100 were added indicated maximum lysis. Specific lysis was determined as: (mean sample c.p.m. – mean spontaneous c.p.m.)/(mean maximum c.p.m. – mean spontaneous c.p.m.)

RESULTS

Coexpression of MUC1 and B7.1 in a mouse mammary cell line inhibts tumour growth in MUC1 transgenic mice

The tumour cell line used to express the MUC1 antigen is the 410.4 mammary epithelial cell, originally cultured from a mammary tumour developing in a BALB/c mouse cross-fostered on a mouse mammary tumour virus (MMTV)-carrying mouse strain.[18] The E3 cell line derived from the 410.4 cell line expressing MUC1 and hygromycin (used for selection) and the control 410.4 transfectant expressing only the selectable marker (410.4-H) have been previously described.[14] These two cell lines were transfected with the mouse B7.1 gene together with the neomycin resistance gene or only with the selectable marker. The cell lines developed and their nomenclature is shown in Fig. 1. As shown in Fig. 2(a), the three cell lines expressing both MUC1 and B7.1 express different levels of B7.1 with E3-B7(Z) showing the highest level. Figure 2(a) also shows that the level of expression of B7.1 is comparable in the E3-B7(Z) cell line and in 410.4-HB7 cells not expressing MUC1. Moreover, the levels of expression of MUC1 in the B7.1 tranfectants are the same as those in the E3 neo transfectant (see Fig. 2b). The growth rates of all of the cell lines *in vitro* were not significantly different (data not shown).

The tumorigenicity of the cells lines was tested in the *MUC1* transgenic mice and Fig. 3 shows the Kaplan–Meier survival curves for mice given the four cell lines 410.4-H.neo, 410.4-B7, E3-neo amd E3-B7(Z) at three different doses (10^5 (Fig. 3a), 2×10^5 (Fig. 3b), 10^6 (Fig. 3c)). Figure 4 shows the growth rates for the individual tumours at the higher dose. The data of Fig. 3 show that the expression of the costimulatory molecule together with the MUC1 antigen has a highly significant effect on survival at the two lower challenge doses, while expression of either B7.1 or MUC1 alone has no such effect. Moreover, even though none of the mice survived the challenge dose of 10^6 cells per mouse, there was a strongly significant effect of coexpression of B7.1 and MUC1 on the growth rate of the tumours resulting in a marked delay in the time of death (Fig. 4).

Influence of level of expression of B7.1

The influence of the level of expresson of the B7.1 molecule was examined by following the development of tumours in groups of mice injected with the three cell lines showing different levels of B7.1 expression (see Fig. 2a). Figure 5 shows the Kaplan–Meier survival curve for the mice in the three groups as compared to that for mice given the cell line expressing only MUC1 (E3-neo). Because the strongest effects were seen with the high-expressing E3-B7(Z) cell line, this line was used in subsequent experiments.

Growth of the cell lines in nude mice

The growth of the cell lines was examined in BALB/c nu/nu mice and Fig. 6 shows the survival curves (a) and the growth curves for the individual mice (b). It can be seen that expression of MUC1 with or without B7.1 at any level has no effect on the growth of the 410.4 tumour cell line in these mice. These results suggest that natural killer (NK) cells, which are found in high levels in nude mice, are not responsible for the decrease in tumorigenicity of the E3-B7 cells seen in the MUC1 transgenic mice.

T-cell responses

Attempts were made to isolate cytotoxic T cells from mice injected with E3-B7(Z) cells, using the syngeneic cell line P815

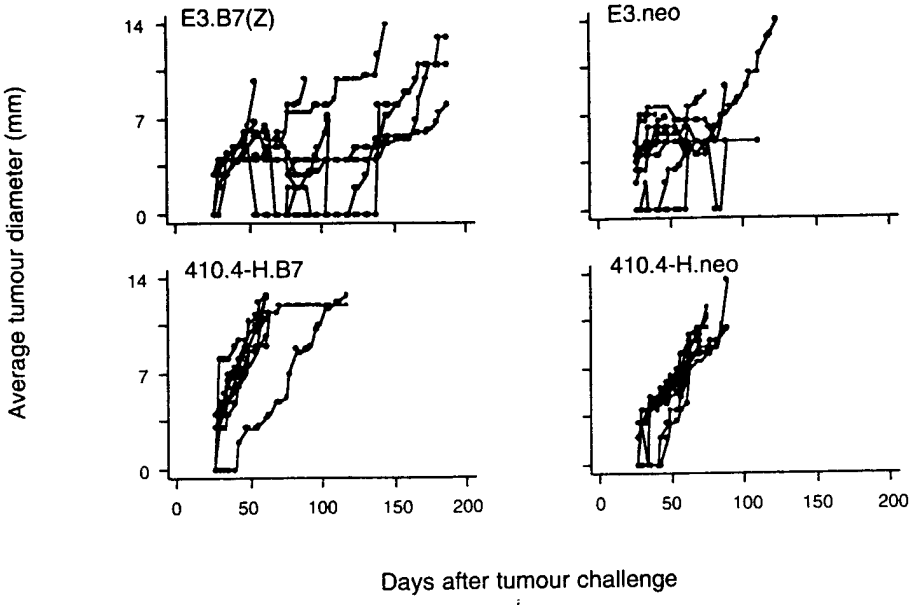

Figure 4. Effect of coexpression of MUC1 and B.7·1 on tumour growth in mice given 10^6 cells (experiment shown in (c). Ten mice were injected in each group. Using the Kruskal–Wallis test for equality of populations, $P=0{\cdot}0001$.

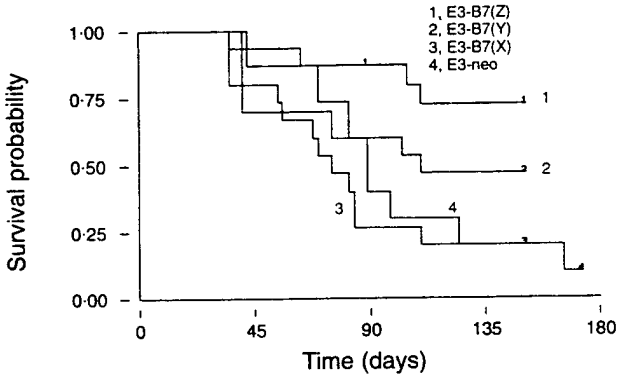

Figure 5. Influence of level of expression of B7.1 on the survival of *Sac*II × BALB/c F1 hybrid mice (15 mice per group) injected with 10^5 E3 cells expressing different levels of B7.1. Log rank test for heteogeneity shows $P=<0{\cdot}01$.

expressing MUC1 to stimulate T cells from the spleens of the injected mice. A low level of specific killing was observed when the same cell line was used as a target. Although the lysis seen was not very high, the killing observed was specific because untransfected P815 cells were not killed. An example of this effect is shown in Fig. 7.

A synthetic peptide covering three tandem repeats was used to measure antibodies in the serum of mice given the various cell lines. The strongest and most consistent antibody response was seen in the mice given the higher doses (10^6 cells) of the E3-B7(Z)cells (data not shown). However, at the lower doses the antibody response measured in this way was not consistent.

In vivo depletion of CD4$^+$ and/or CD8$^+$ cells stimulates tumorigenicity of E3-B7(Z) cells in the MUC1 transgenic mice

To assess the importance of T-cell responses *in vivo* in the inhibition of tumorigenicity induced by 410.4 cells expressing

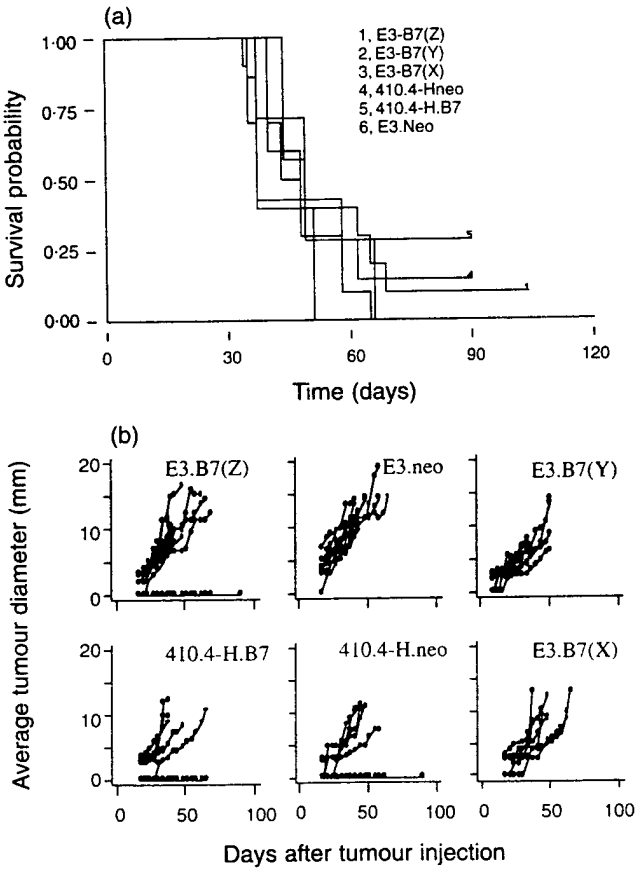

Figure 6. Growth of 410.4 derived lines in nu/nu mice. Cells (10^5) from each of the cell lines indicated were injected into the mice, and the development of individual tumours followed. (a) Survival curves; (b) growth of individual tumours. The log rank test for heterogeneity of the survival curve gives a probability of 0·86, and the Kruskal–Wallis test for equality of populations gives a probability of 0·98.

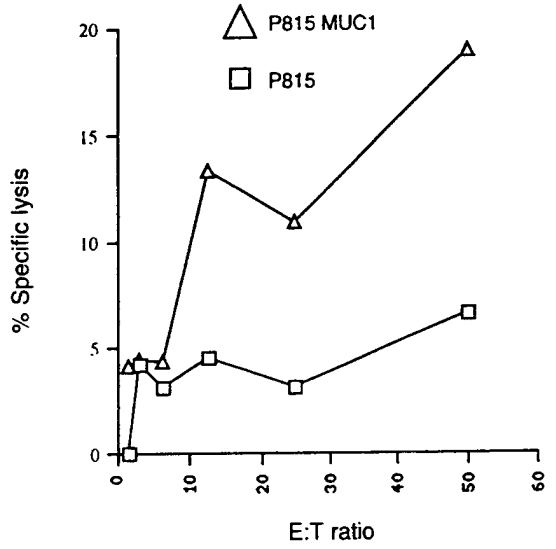

Figure 7. F1 hybrid mice were injected with 1×10^5 E3-B7(Z) cells and after 4 weeks spleens were removed, stimulated with the P815 MUC1 cell line and CTL responses to MUC1 were analysed. Killing was measured in a chromium release assay.

both MUC1 and B7, mice were depleted of $CD4^+$ or $CD8^+$ cells or of both subclasses by injecting antibodies to the CD4 or CD8 molecules after thymectomy (see Materials and Methods). Control mice were thymectomized and treated with an irrelevant antibody. The survival curves and growth of individual tumours are shown in Fig. 8(a,b). Although thymectomy itself showed some inhibitory effect on the ability of the mice to resist the growth of the tumour cells, the effect of depletion of either $CD4^+$ cells or $CD8^+$ cells had a much more pronounced effect, and survival was decreased and rate of tumour growth increased. The effect on both survival and growth rate was marginally stronger in the CD4 depleted mice, however, the mice depleted of both $CD4^+$ and $CD8^+$ cells showed the worst survival. These results demonstrate unequivocally that the protection afforded by expression of B7.1 together with the MUC1 antigen requires stimulation of specific $CD8^+$ and $CD4^+$ T cells.

Enhancement of immunogenicity does not affect normal MUC1-expressing epithelial cells

The MUC1 mucin is expressed on most glandular epithelial cells including the pancreas, stomach, lung, kidney and the lactating breast, and this pattern of expression is faithfully reproduced in the MUC1 transgenic mice. The mice injected with the E3-B7(Z) cells survived their natural life span and tissues were taken for examination at monthly intervals after being injected with the tumour cells. An examination of the normal glandular epithelial tissues expressing MUC1 showed no evidence of abnormalities, confirming that the immune response that prevented the growth of the tumour cells did not adversely affect the normal tissues.

DISCUSSION

Several clinical studies are in progress using MUC1-based vaccines, mostly in breast cancer patients with advanced

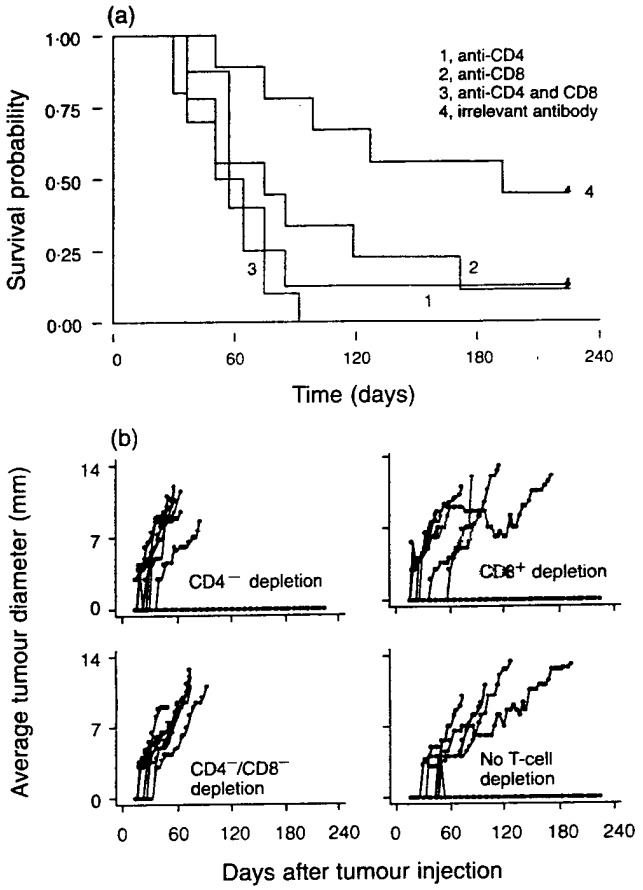

Figure 8. *In vivo* depletion of $CD4^+$ and $CD8^+$ T cells abolishes protective effect of B7.1 expression on tumourigenesis. F1 *Sac*II BALB/c mice were thymectomized and treated with antibodies to CD4, CD8, to both antigens or with an irrelevant antibody, as described in materials and methods, before challenging with 10^5 E3–B7(Z) cells.The survival of the mice (a), and development of tumours (b) was monitored.Using the log rank test for heterogeneity $P = 0{\cdot}027$.

disease.[9] Preclinical testing of these vaccines in model systems should give an indication of both efficacy and potential toxicity, which in this case could relate to autoimmune responses, because the antigen is expressed on normal epithelial tissues. In this paper we have used transgenic mice expressing the human MUC1 mucin as a self antigen to determine if an effective immune response can be generated without the induction of autoimmunity. The data show that coexpression of the MUC1 antigen with B7.1 in a mouse tumour cell line dramatically reduces the tumorigenicity of the cells in the MUC1 transgenic mice without inducing abnormalities in the MUC1 expressing tissues, or affecting the life span of the injected mice.

The MUC1 antigen is normally expressed on the luminal surface of most glandular epithelial cells, but is up-regulated and aberrantly glycosylated in breast and other carcinomas.[1] The molecule is highly repetitive with an extended structure due to the addition of multiple *O*-glycans to serines and threonines found in the tandem repeat sequence. In cancer, the *O*-glycans are shorter[22] so that core protein epitopes are exposed which are normally masked and novel carbohydrate epitopes appear. A similar change appears to occur in mice.

because the same epitope in the tandem repeat recognized by the antibody SM3[23] is masked in normal tissue in the MUC1 transgenic mouse[13] but exposed in tumours developing in these mice.[15]

The changes in glycosylation will affect any immune response which depends on the interaction of the whole MUC1 molecule with an effector cell. This would apply to the induction of antibodies, and possibly to the induction of any MHC unrestricted T-cell responses which have been reported to be specific for the cancer-associated mucin in humans.[5] Moreover, interactions with surface lectins on antigen-presenting cells (APCs) and consequently antigen uptake will also depend on the glycoform, as will the breakdown in the APC[24,25] and the presentation by MHC class II molecules.[26] There are, therefore, multiple ways of recruiting immune responses to MUC1 in addition to the classic stimulation of T cells by specific peptides presented by MHC molecules, which however, have also been described.[7,8,27] It was therefore of interest to ask whether the immune responses which lead to effective tumour rejection are those which require the costimulatory signals generated by the interaction of B7.1 with T cells. Our results clearly show that expression of B7.1 together with the MUC1 mucin in a mouse mammary tumour cell dramatically enhances its immunogenicity and that the immunity requires the activity of $CD8^+$ and $CD4^+$ T cells. Overexpression of B7.1 in the absence of MUC1 had no significant effect on the immunogenicity of the mammary epithelial tumour cells (410.4) used in the experiments. Analogous to the report of Cayeux *et al.*,[28] the data suggest that the MUC1 antigen requires costimulation generated by the B7.1–T-cell interaction to induce an effective response. Alternatively, antigen presentation could occur through cross-priming of tumour-specific T cells stimulated by B7.1 expression.[29] Using a different model system, Gong *et al.* have recently shown that immune responses to MUC1 can be induced by immunizing MUC1 transgenic mice with dendritic cells fused with MUC1-expressing carcinoma cells.[30] The use of professional APC in this system also suggests a role for costimulatory molecules in the effective presentation of MUC1.

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